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This international journal publishes original research and critical reviews in agriculture and food science, with particular emphasis on interdisciplinary studies at the agriculture/food interface. Its remit embraces fundamental and applied research and development of the production of crops and animals (including fish), comprising: studies of plant and animal husbandry, physiology, yield and quality, and the interaction of agriculture with forestry and the environment; their importance in human food, fibre and animal feed; the nutritional quality and sensory analysis, flavour, texture, safety and toxicity of important human foodstuffs, beverages and alcoholic drinks. Research papers should not exceed 3000–4000 words together with relevant data. Excessively long contributions are likely to be subjected to delay.

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Traffic-induced Compaction in Maize, Cowpea and Soya Bean Production on a Tropical Alfisol After Ploughing and No-tillage: Crop Growth

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(Manuscript received 11 April 1986)

An experiment was conducted over three consecutive seasons during 1982 and 1983 to evaluate the effects of traffic-induced compaction on growth and yield of maize, cowpea and soya bean grown in a tropical Alfisol for no-tillage and ploughed systems. Compaction treatments of no, two and four passes of a 2-tonne roller were used on the sub-plots. The roller was 60 cm in diameter, 180 cm long, with a contact area of 0.1729 m^2 and was pulled by a 33.6 kW tractor. The four-pass treatment reduced percentage emergence, plant height, leaf number, leaf area index, and dry matter yield of maize, cowpea and soya bean. These reductions were more marked on ploughed than on no-till plots. The two- and four-pass treatments significantly reduced root growth of maize, cowpea and soya bean in the 0–70 mm depth, but the reduction in root density was greater in ploughed than in no-tillage. The two- and four-pass compaction treatments reduced plant nutrient uptake and grain yield of maize, cowpea and soya bean. The four-pass treatment reduced maize grain yield by 48% in all seasons under no-tillage and by 53, 61 and 75% after ploughing in the first, second and third consecutive cropping seasons respectively, compared to the yield of the relevant non-compacted treatment. Cowpea yield was reduced by 38% in all seasons after no-tillage and by 39, 50 and 57% after ploughing during the first, second and third consecutive cropping season respectively. The response of soya bean to four-pass compaction resulted in yield reduction by 50, 64 and 25% after no-tillage and by 47, 48 and 65% after ploughing in the first, second and third consecutive cropping season, respectively. Crop yields were found to be negatively correlated with soil bulk density, penetrometer resistance, and relative compaction, and positively correlated with infiltration rate, soil moisture content and specific volume. Multiple regression equations were also developed between crop yield and soil properties.

Keywords: Tropical soils; crop growth; root growth; soil moisture; soil compaction; soil physical properties.

1. Introduction

Mechanical land clearing in tropical Africa has been shown to cause compaction of the surface soil horizons.[1–3] Soil compaction problems in this region are further accentuated by intense tropical rain storms,[4–5] the presence of naturally compacted soil layers,[7, 8] and the widespread occurrence of soil horizons with a large percentage of gravel.[9–11] Large concentrations of gravel *per se* can have significant adverse effects on the root system development of maize[12–14] and on crop growth by diluting both water and nutrient intensity and capacity factors.[15]

While soil compaction resulting from increased use of large machinery and more intense cultivation[16–19] has been shown to hamper significantly the performance of various crops in temperate regions, very little detailed information is available in tropical Africa. In East Africa, Pereira and Jones[20] demonstrated the disadvantages of progressive soil structural deterioration with ploughing and discing in successive seasons. In Nigeria, Lal[21] observed severely adverse effects of mechanised

farm operations on soil physical properties and maize grain yield. Many other soils of the tropics are rapidly being brought under mechanised cultivation for food crop production but information is lacking on soil and crop responses.

The adverse effects of traffic-induced compaction on soil physical properties were described in an earlier report.[22] This report describes the effects of compaction on growth and yield of maize (*Zea mays*), cowpea (*Vigna unguiculata*) and soya bean (*Glycine max*). These experiments were conducted at the same sites in the same seasons for which the soil physical properties have been described in an earlier report.[22]

2. Experimental

2.1. Site description

There are two growing seasons in this ecological zone: April–July and September–November. The mean rainfall for each season is about 800 and 400 mm, respectively. There is a long dry season from mid-November to mid-March. The experiment was conducted in three consecutive rainy seasons during 1982–1983 at the experimental farm of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The rainy seasons were the second season in 1982 and the first and second seasons in 1983. The experiment was set up as a split plot design with tillage treatments (ploughing and no-tillage) as main plots and levels of induced compaction as sub-plots. The sub-plot treatments consisted of nought, two and four passes of a 2-tonne roller pulled by a 33.6 kW tractor. The roller was 60 cm in diameter, 180 cm long, and was used to stimulate normal field traffic in the weight range of 1.5–2.5 tonnes. The contact area between the roller and soil was 0.1729 m^2. Thus the contact pressure exerted by the roller on the soil was 113 kPa per pass. The ploughing treatment consisted of disc ploughing to a depth of 200 mm followed by harrowing compared to no mechanical seedbed preparation for the no-tillage system. Prior to this experiment, the soil was ploughed twice every year for growing one crop of maize every season since 1971. The no-tillage plots were sprayed, manually using a knap-sack sprayer, with paraquat (1-1′ dimethyl-4, 4-bipyridylium ion) at the rate of 2.5 litres ha^{-1}. Compaction treatments were then initiated on both ploughed and no-tillage plots. The experiment was replicated three times.

2.2. Experimental methods

Descriptions of methods of measuring soil physical properties are given in part I of this study.[22] Crops were planted manually using a dibbler at a row spacing of 75 cm and plants spaced at 25, 25, and 5 cm, for maize (*Zea mays* L.) cowpea (*Vigna unguiculata* [L.] Walp) and soya bean (*Glycine max* Merr.) respectively. Fertiliser application consisted of 120 kg ha^{-1} N (40 kg at planting and 80 kg 4 weeks later) as urea for maize; 26 kg ha^{-1} P as single superphosphate and 30 kg ha^{-1} K as potassium chloride for maize, cowpea and soya bean. Cowpea and soya bean received no N fertiliser. Crops were sown on 17 September 1982 (second season), 13 April 1983 (first season) and 13 September, 1983 (second season).

Seedling emergence was determined in each experimental unit until no additional germination occurred. The number of leaves per plant and plant height were determined fortnightly. Leaf area (for leaf area index determination) was measured fortnightly by means of a leaf-area meter. Dry matter production was determined fortnightly by oven-drying harvested green material at a temperture of 60°C before weighing. During the first season (1983) root samples were taken by the core method at fortnightly intervals in 70 mm depth increments down to 210 mm from the area where shoots had recently been harvested. These samples were saturated overnight and then washed with a gentle spray of water over a 2-mm sieve. Washed roots were picked up by forceps, rewashed and then dried at 60°C. Root density was expressed as the weight of dry roots per unit volume of soil (mg cm^{-3}). At the early flowering stage, leaf samples were taken, dried at 60°C for 48 h and ground for elemental analysis. At maturity the crops were harvested manually, shelled and grain yield recorded at 10% moisture content.

3. Results and discussion

3.1. Emergence

Percentage emergence of maize, cowpea, and soya bean as affected by tillage and compaction treatments for three consecutive seasons is shown in Table 1. With the exception of soya bean, significant differences in percentage emergence between compacted treatments only occurred in ploughed plots. The two- and four-pass compaction treatments reduced the percentage emergence of soya bean, but the reduction was more drastic in ploughed than in no-tillage plots.

3.2. Plant growth and dry matter production

Differences in plant height of maize and soya bean between compacted and non-compacted treatments increased markedly in the first and second season in 1983, although the data are shown for one season only (Figure 1). At 8 weeks after planting (WAP) during the second season, for example, the two-pass treatment reduced plant height by 35% for no-till and by 47% for ploughing in maize, and by 28 and 38% for no-tillage and ploughing respectively in soya bean. The four-pass compaction treatment reduced plant height by 50% for no-till and 62% for ploughing in maize, and by 37% for no-till and 51% for conventional ploughing in soya bean.

Compaction reduced the leaf number of maize in both no-till and ploughed plots (Figure 2). However, the differences in leaf number between compacted and non-compacted treatments increased considerably in both tillage systems in the third consecutive cropping season (data not shown). At 8 WAP during the second season in 1983, for instance, the mean leaf number for no-compaction, and two- and four-pass treatments was 12.7, 10.3 and 9.5 for no-tillage and 13.3, 9.7 and 8.8 for ploughed seedbed, respectively. The two-pass compaction was as effective as four-pass in reducing the leaf number of cowpea for all three consecutive seasons. The soya bean leaf number was also reduced by compaction, but the effect was more marked in the first and second seasons in 1983. At 8 WAP in the second season in 1983, for example, soya bean leaf number for no-compaction, and two- and four-pass treatments were in the order 13.4, 9.5, and 7.2 for no-tillage and 13.3, 9.3 and 7.7 under conventional ploughing.

Compaction reduced the leaf area of maize, cowpea and soya bean (Figure 3), but the severity of leaf area reductions increased in the third consecutive cropping season. The greatest reduction in leaf area was observed in the four-pass treatment on ploughed plots. At 8 WAP during the second season in 1983, for example, the leaf area index of the four-pass treatment was reduced by 44 and 68% in maize, by 60 and 85% in cowpea and by 61 and 79% in soya bean under no-tillage and ploughed plots, respectively. Reduced leaf area index in compacted treatments resulted from reductions in leaf number rather than reduced leaf size.

Reduction of dry matter yield by either two- or four-pass compaction was more marked in the third consecutive cropping season in 1983 than in earlier seasons (Figure 4). For instance, the four-pass compaction treatment reduced dry matter yield by 48 and 70% in maize, by 77 and 76% in soya bean and at 8 WAP by 73 and 77% in cowpea under no-tillage and ploughing treatments, respectively. The non-compacted but ploughed plot produced the highest dry matter yield due to relatively low dry bulk densities and high total porosities.[22]

3.3. Leaf nutrient concentration

There were generally no significant differences in plant nutrient content among compaction treatments in both no-till and ploughed plots at tasseling stage of maize in the second season 1982 and first season 1983 (Table 2). In the second season 1983, however, the four-pass treatment significantly reduced maize leaf nutrient content of P, Ca, Mg, K and Mn in both no-till and ploughed plots. Plant nutrient content in cowpea was affected by compaction treatments in the second season 1983 when two- and four-pass treatments reduced the content of Ca and Mg in no-tillage plots. Similarly, plant nutrient content of N and Ca in soya bean was reduced by the two- and four-pass compaction treatments in ploughed plots during the second season 1983.

Table 1. Percentage emergence of maize, cowpeas and soya beans in varying tillage and compaction treatments

Treatment	Emergence (%)								
	Maize			Cowpeas			Soya beans		
	September 1982	April 1983	September 1983	September 1982	April 1983	September 1983	September 1982	April 1983	September 1983
00[a] NT[b]	81.8a	87.8a	84.5ab	73.5a	84.5ab	90.1a	72.5b	90.0b	86.0b
02 NT	77.8a	91.7a	88.6a	70.6a	89.2a	95.3a	69.3b	79.0c	76.0c
04 NT	66.0b	85.9a	79.1b	69.5a	88.4a	88.2ab	62.9c	80.3c	73.0c
00 CT	86.5a	88.2a	90.7a	69.1a	84.5ab	92.7a	89.7a	95.0a	92.0a
02 CT	83.2a	58.6b	72.9c	58.4b	87.2a	81.9b	74.1b	68.1c	59.1d
04 CT	79.3a	55.1b	74.9bc	46.6c	79.5b	77.9b	68.4bc	55.0d	50.2e

a–e, Letters denote significance at 5% level using Duncan's new multiple range test. Means with the same letter are not significantly different.

[a]Number of passes of a 2-tonne roller.

[b]Tillage treatment: NT=no-tillage, CT=conventional tillage.

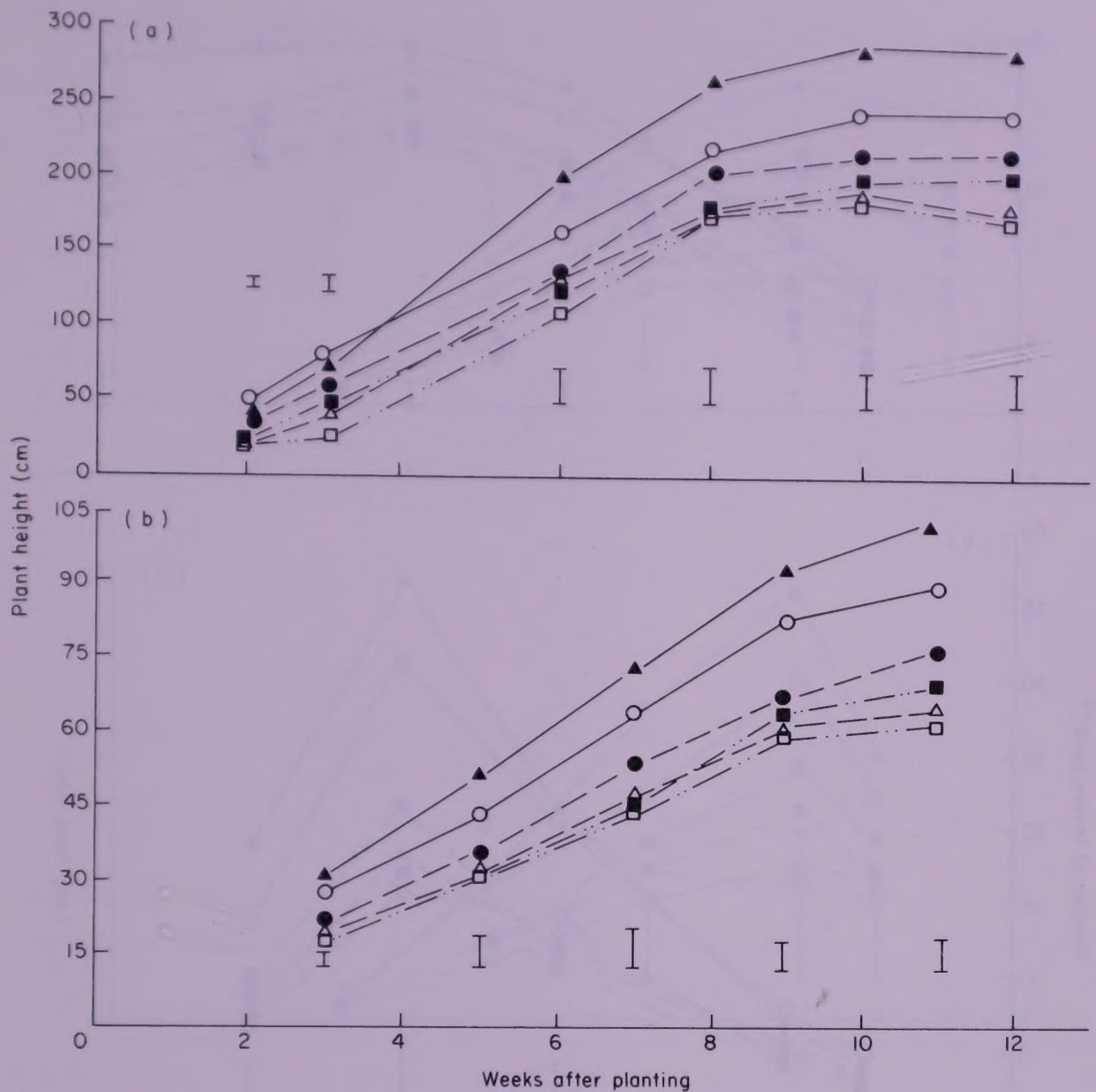

Figure 1. Plant height of (a) maize and (b) soya beans as affected by different soil compaction treatments. No-tillage treatment: (○—○), no-pass; (△--△), two-pass; (□-·-□), four-pass. Conventional tillage: (▲—▲), no-pass; (●--●), two-pass; (■-·-■), four-pass. Bars show standard deviation.

3.4. Root growth

Root growth of maize monitored in the first season 1983 is shown in Figure 5. There were significant differences in root growth of maize between the no-, two- and four-pass treatments for both no-tillage and conventional tillage at all depths. The highest root density, however, was observed in the top 0–70 mm soil layer. There was a sharp decline in root density with soil depth. This was partly caused by an observed increase in gravel concentration with depth. Earlier studies in the vicinity of the experimental site showed that root growth of maize was significantly reduced by increased gravel concentration with depth.[12, 23] In addition to the impeding effect of gravel layer, traffic-induced compaction partly caused reduction in maize root density with soil depth as was also reported by other researchers.[19]

The root density of cowpea was markedly higher in non-compacted than in two- or four-pass treatments under both tillage systems (Figure 6). Unlike maize, the decline of root density with increase in depth was very gradual. This was due to the deep rooting habit of cowpea, the root of which have been shown to penetrate into soil having high bulk density and strength.[24, 25]

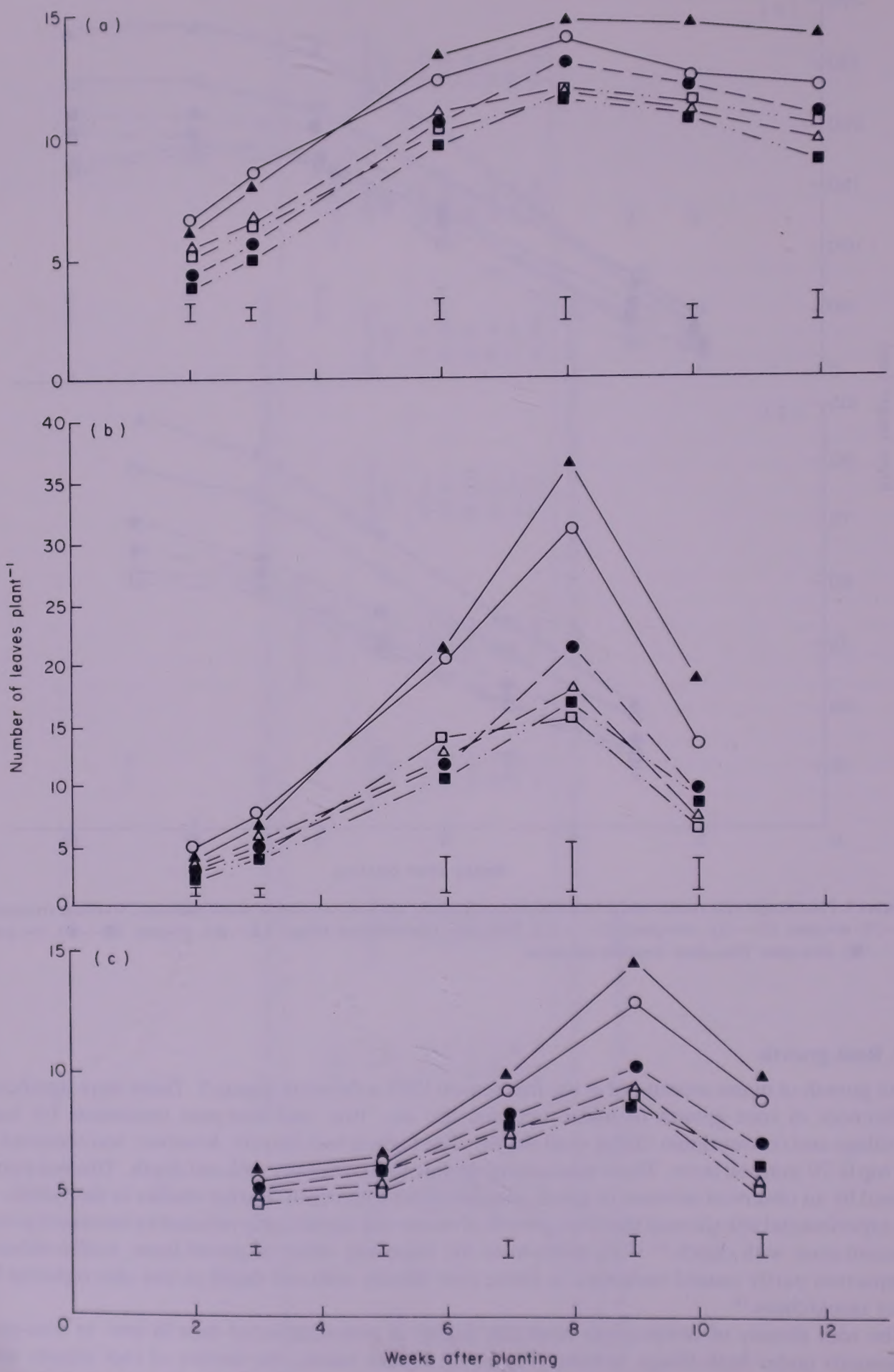

Figure 2. Effect of soil compaction treatments on number of leaves per plant for (a) maize, (b) cowpeas and (c) soya beans. For symbols see Figure 1.

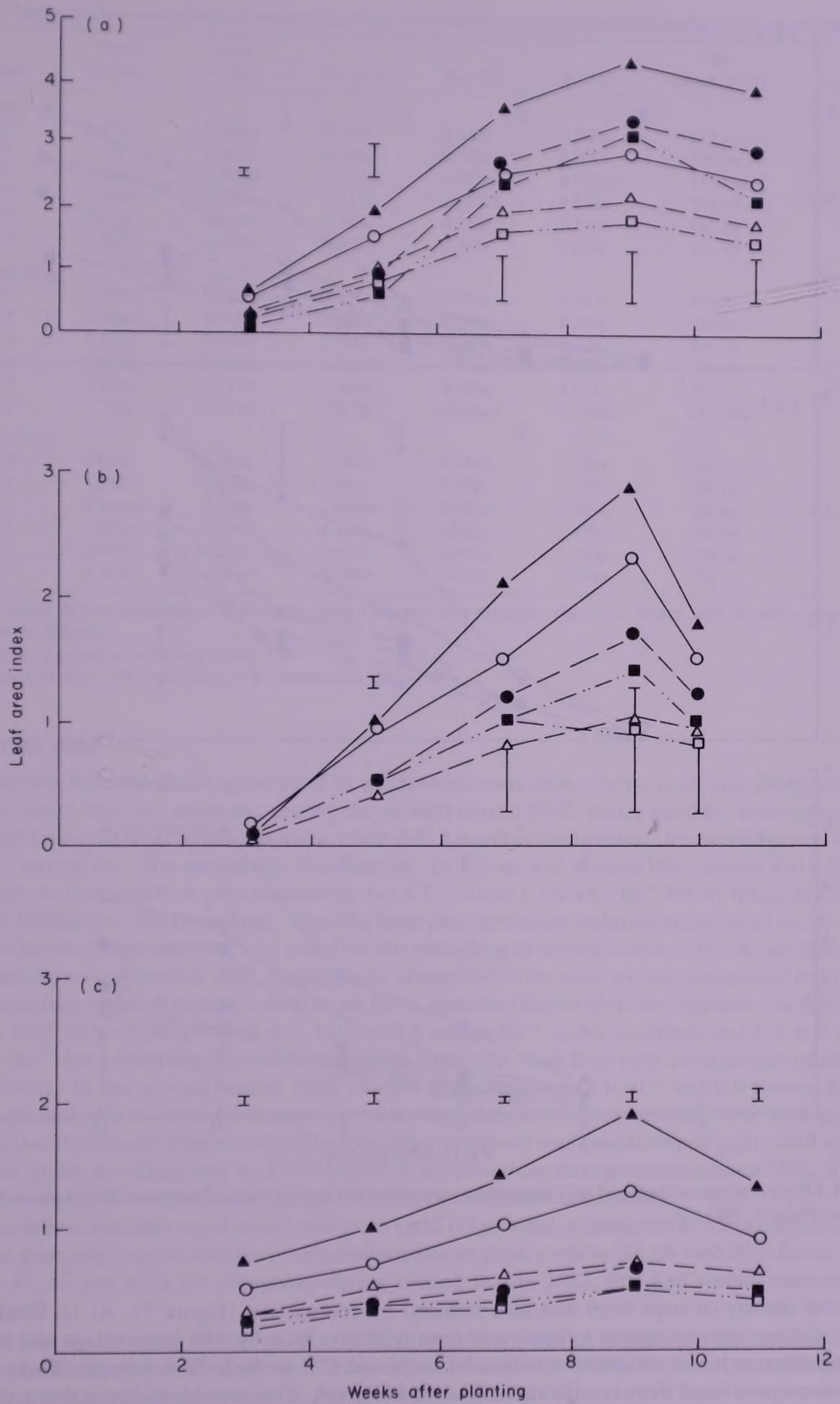

Figure 3. Leaf area index of (a) maize, (b) cowpeas, and (c) soya beans as influenced by compaction and tillage treatments. For symbols see Figure 1.

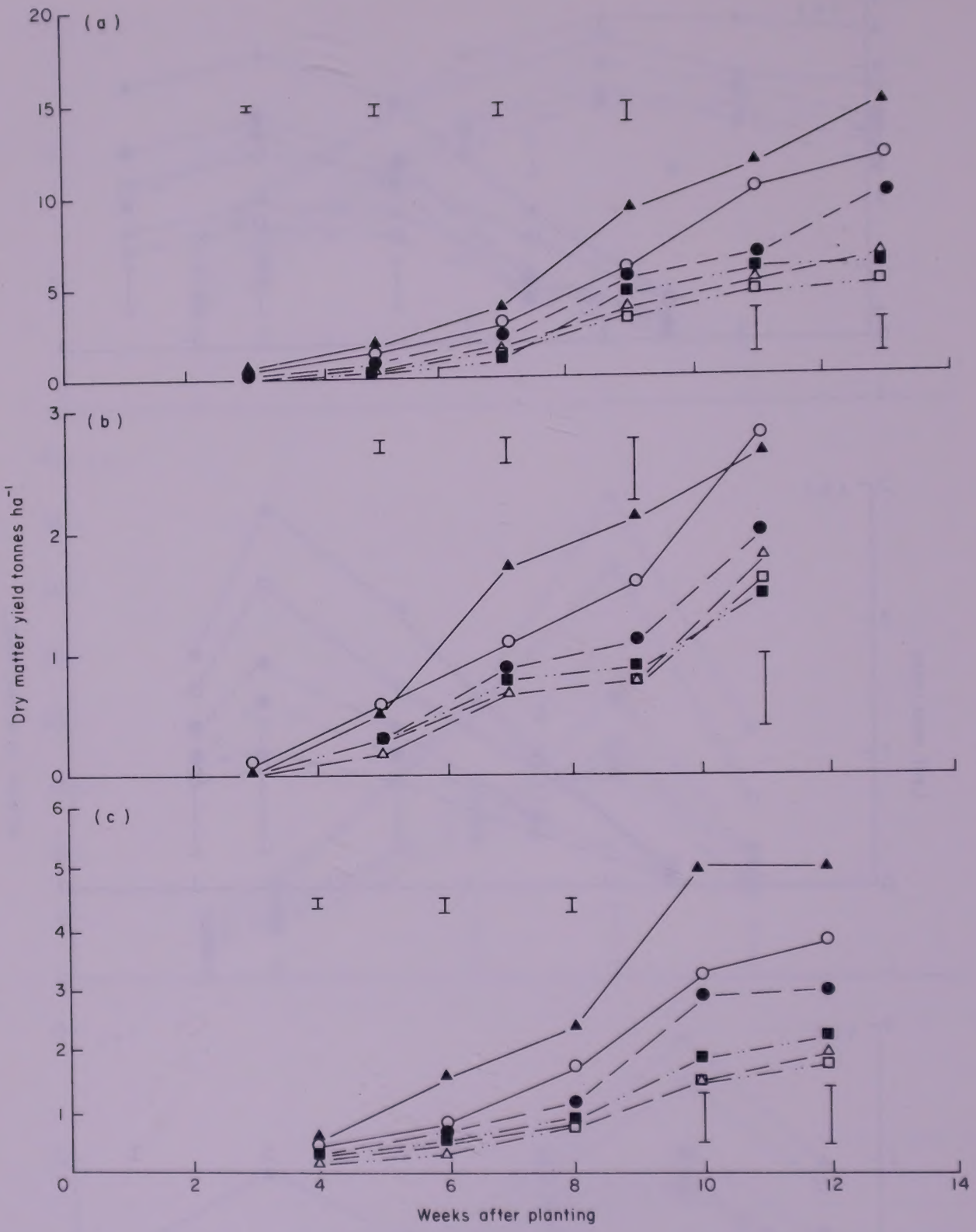

Figure 4. Effects of tillage methods and soil compaction on dry matter yields of (a) maize, (b) cowpeas and (c) soya beans. For symbols see Figure 1: 5% l.s.d.

The root density of soya bean was also reduced by compaction (Figure 7). At 10 WAP, for example, the two-pass treatment reduced root density of soya bean by 30% in no-tillage and 58% in ploughing whereas heavy compaction reduced it by 54 and 67% in the 0–70 mm depth. There was a marked decrease of root density with an increase in soil depth. This was due to the inability of soya bean roots to penetrate into soil having high bulk density and strength resulting from high concentrations of gravel at depth.[23]

Table 2. Leaf nutrient concentration of maize at tasselling, and cowpeas and soya beans at flowering in the second season 1983

Treatment		Total N (%)	Total P (%)	Ca (%)	Mg (%)	K (%)	Mn (parts 10^{-6})	Zn (parts 10^{-6})
(a) Maize								
00[a]	NT[b]	2.841a	0.390a	0.711a	0.325a	5.366a	213.1a	58.3a
02	NT	2.13a	0.348a	0.706a	0.340a	6.208a	190.4a	39.5a
04	NT	2.251a	0.315ab	0.513ab	0.264bc	4.713ab	177.5ab	48.0a
00	CT	2.944a	0.323a	0.524a	0.310ab	4.876a	178.9a	38.7a
02	CT	2.452a	0.288b	0.545a	0.273b	5.546a	167.3b	40.8a
04	CT	2.548a	0.266b	0.420b	0.245c	4.600b	160.1b	43.3a
(b) Cowpeas								
00[a]	NT	3.480a	0.212b	3.194a	0.466a	4.033b	478.2a	29.7a
02	NT	3.206a	0.216b	2.521b	0.400b	4.292a	611.0a	34.1a
04	NT	3.185a	0.285a	2.440b	0.393b	4.426a	556.7a	35.1a
00	CT	3.674a	0.232ab	2.823ab	0.474a	4.763a	353.4b	30.6a
02	CT	3.699a	0.219b	2.396b	0.438a	4.071b	322.1b	35.8a
04	CT	3.727a	0.218b	2.372b	0.436ab	4.134ab	437.9ab	36.2a
(c) Soya beans								
00[a]	NT[b]	2.982b	0.202a	1.282a	0.444a	2.548a	206.6a	35.0b
02	NT	3.256b	0.195a	1.091a	0.499a	2.762a	199.1a	39.8b
04	NT	3.405ab	0.200a	1.049ab	0.442a	2.903a	180.8a	33.6b
00	CT	4.053a	0.175a	1.111a	0.441a	2.435a	180.6a	37.9b
02	CT	2.940b	0.202a	0.973a	0.386a	2.824a	200.4a	40.6b
04	CT	3.142b	0.183a	0.989b	0.428a	2.899a	220.1a	51.3a

a–c, Letters denote significance at 5% level using Duncan's new multiple range test. Means with the same letter are not significantly different.

[a]Number of passes of a 2-tonne roller.

[b]Tillage treatment: NT=no tillage, CT=convention tillage.

3.5. Grain yield

Compaction reduced maize grain yield in all three seasons under both no-till and ploughed treatments (Figure 8a). For example, during the second season 1982, maize yield for non-compaction, two- and four-pass treatments was in the order 5.9, 3.8 and 2.9 tonnes ha^{-1} for no-tillage and 6.9, 4.6 and 3.3 tonnes ha^{-1} for ploughing, respectively. In the second season 1983, maize yield for non-compaction, two- and four-pass treatments was 4.7, 3.0 and 2.3 tonnes ha^{-1} for no-tillage and 5.4, 1.6 and 1.4 tonnes ha^{-1} for ploughing. Thus the four-pass treatment reduced maize yield by 51, 41 and 52% under no-tillage and by 53, 61 and 75% for ploughing in second season 1982, in the first season 1983 and the second season 1983, respectively, compared to the yield of non-compacted treatments.

Compaction reduced cowpea yield in all three seasons (Figure 8b). For instance, in the second season 1983 yield of cowpea was 1.3, 1.0 and 0.8 tonnes ha^{-1} under no-tillage and 1.3, 0.9 and 0.8 tonnes ha^{-1} for ploughing for non-compaction, and two- and four-pass compaction treatments, respectively. In the second season 1983 cowpea grain yield was 1.0, 0.7 and 0.6 tonnes ha^{-1} for no-tillage and 1.2, 0.7 and 0.5 tonnes ha^{-1} for ploughing for non-compacted, two- and four-pass compaction treatments respectively. The four-pass treatment decreased cowpea grain yield by 34, 44 and 36% under no-tillage and by 39, 50 and 57% for ploughing during second season 1982, first and second season 1983 respectively, compared to non-compacted treatments.

Compaction also decreased soya bean grain yield (Figure 8c). Compared to non-compacted plot, the four-pass compaction treatment reduced soya bean grain yield by 50, 64, and 25% for no-tillage and by 47, 47 and 65% for ploughing during second season 1982, first and second seasons 1983, respectively.

Generally the severity of compaction effects on grain yields of maize, cowpea and soya bean increased with repeated compaction in subsequent seasons, especially in the second season 1983. The effect was particularly severe on ploughed plots. Reduced grain yield from compacted treatments was due to low grain weight per cob for maize, low grain weight per plant for cowpea and reduced unit weight per 100 grains for soya bean (Table 3). Relationships between soil physical

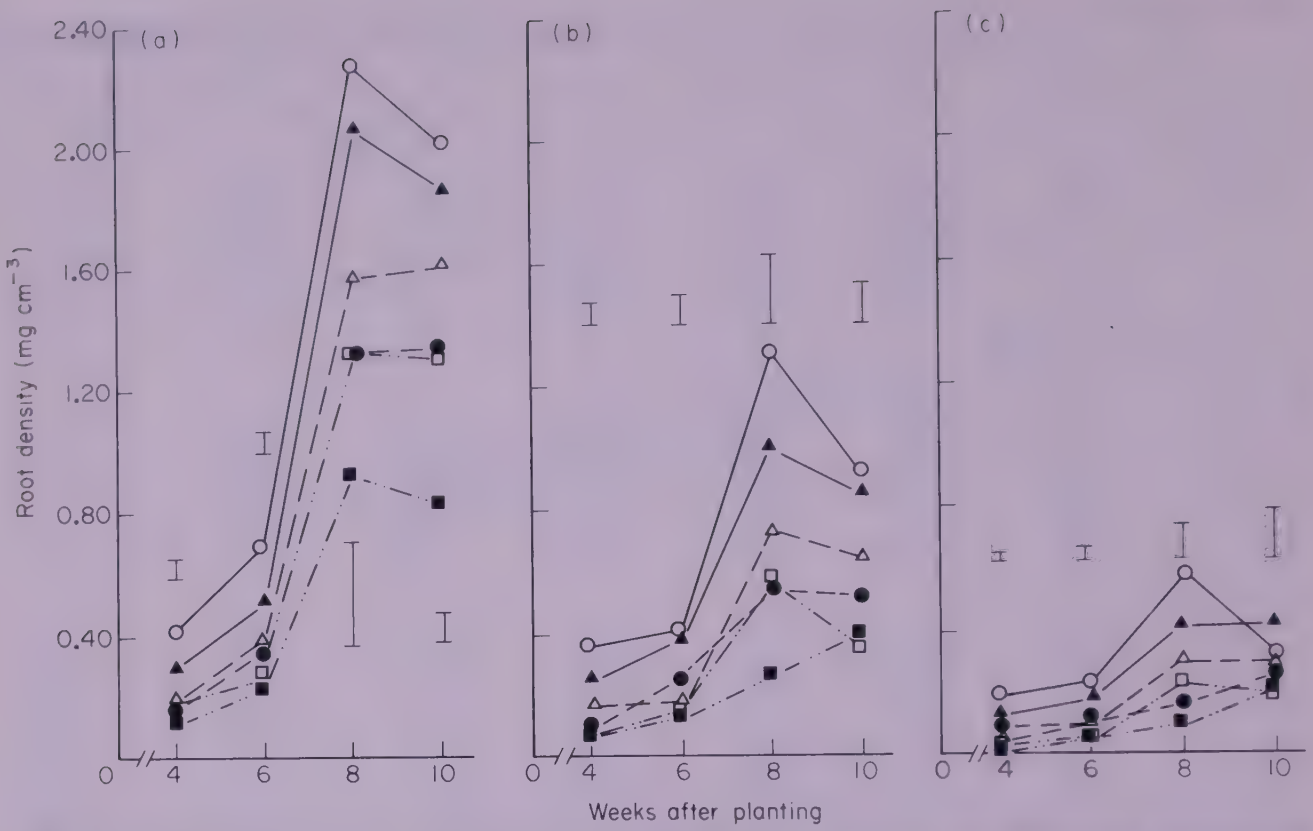

Figure 5. Root weight density of maize as affected by traffic-induced soil compaction and tillage treatments. (a) 0–70 mm, (b) 70–140 mm, (c) 140–210 mm. For symbols see Figure 1: 5% l.s.d.

properties and grain yield are shown in Table 4. Maize grain yield was negatively correlated with dry bulk density, penetrometer resistance and relative compaction in all three seasons. The correlation coefficients were significant at 1% level of probability and ranged from 0.57–0.83. In comparison, maize grain yield was positively correlated with infiltration rate, total porosity, and specific volume. The correlation coefficients ranged from 0.67–0.87. Similar observations have been reported by other workers.[7, 25–28]

Regression analyses between cowpea grain yield and soil physical properties also indicated negative correlations with bulk density, penetrometer resistance and relative compaction. The correlation coefficient were significant at 1% level of probability and ranged from 0.66–0.85. In comparison, grain yield of cowpea was positively correlated with infiltration rate, total porosity and specific volume. These correlation coefficients were also statistically significant at 1% level of probability and ranged between 0.53 and 0.87. Similar results were reported for cowpea by other researchers.[25, 29]

Soil physical properties were also significantly correlated with the grain yield of soya bean. In general, the correlation coefficients were low in the first cropping cycle (second season in 1982) than in the second or the third consecutive crop. The negative correlation coefficients between the grain yield and soil physical properties (bulk density, penetrometer resistance and relative compaction) ranged from 0.55–0.83. In comparison, the range of positive correlation coefficients between soil physical properties (total porosity, infiltration rate and specific volume) and grain yield of soya bean ranged from 0.53–0.93. Soya bean grain yield has also been reported to be correlated with soil physical properties by other researchers.[30–33]

Comparative effects of soil compaction on mean grain yields for three seasons for maize, cowpea and soya bean are shown in Figure 9. The data show that traffic-induced compaction reduced grain yield more in maize than in cowpea and soya bean. Although grain yields of all three crops decreased linearly with increase in bulk density from 1.2–1.8 tonnes m^{-3}, the rate of yield decline was in the order of maize >soya bean >cowpea.

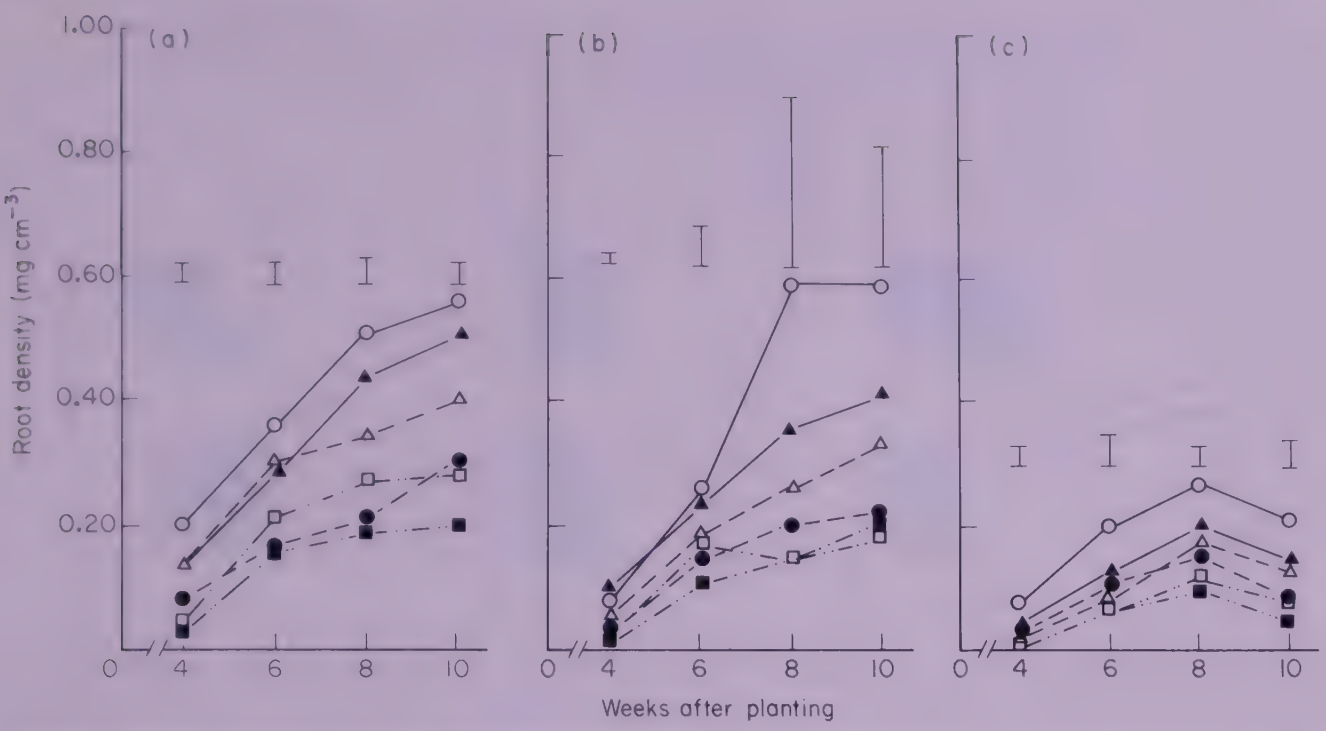

Figure 6. Root weight density of cowpeas for different soil compaction and tillage treatments: (a) 0–70 mm (b) 70–140 mm, (c) 140–210 mm. For symbols see Figure 1: 5% l.s.d.

Agrawal[26] reported that yield reduction of cereal crops due to compaction was less than that of legumes, and suggested that crops having tap root systems were more susceptible to compaction at shallow depths than cereal crops having fibrous root systems. That the above conclusion does not agree with the results reported here could be due to the deep rooting habit of legumes used in these experiments, especially cowpea (Figure 6).

3.6. Characterisation of soil compaction in relation to crop yield

The stepwise regression procedure was used to evaluate the relative effects of different soil parameters influenced by compaction on crop yields. The regression equations derived from such data

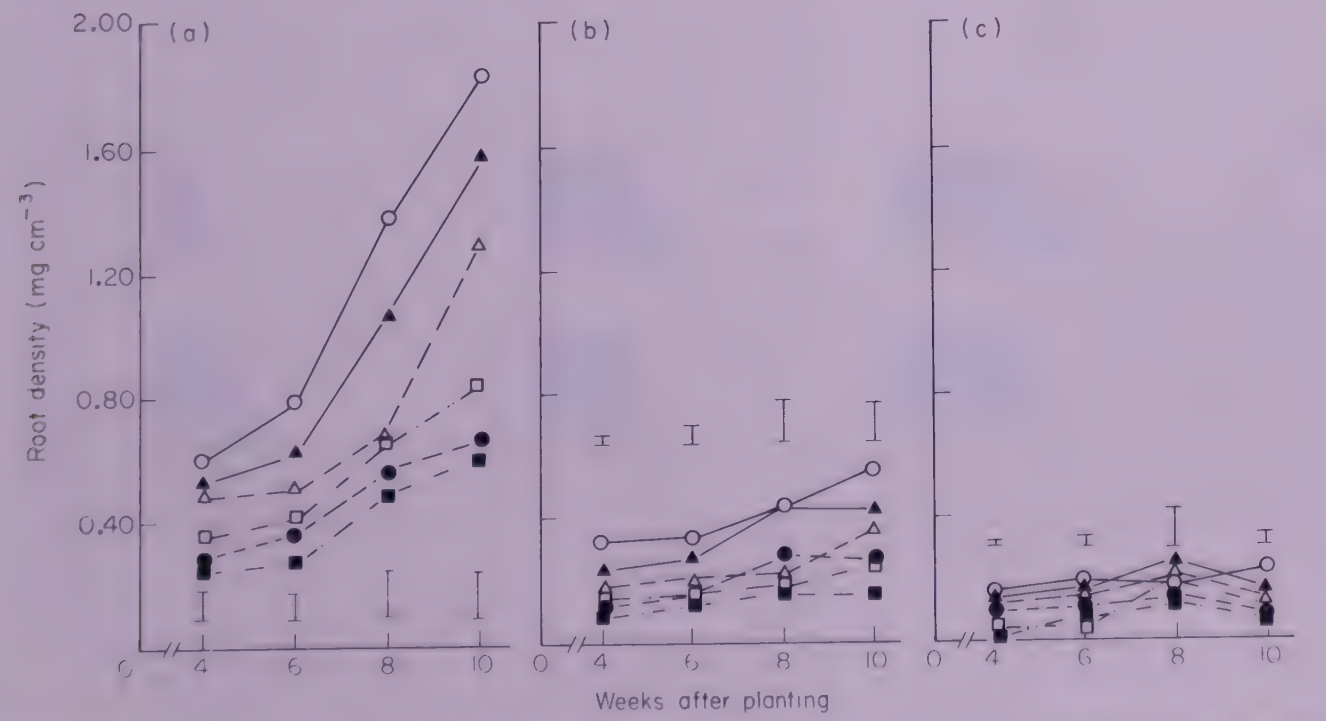

Figure 7. Root weight density of soya beans for different soil compaction and tillage treatments: (a) 0–70 mm, (b) 70–140 mm, (c) 140–210 mm. For symbols see Figure 1: 5% l.s.d.

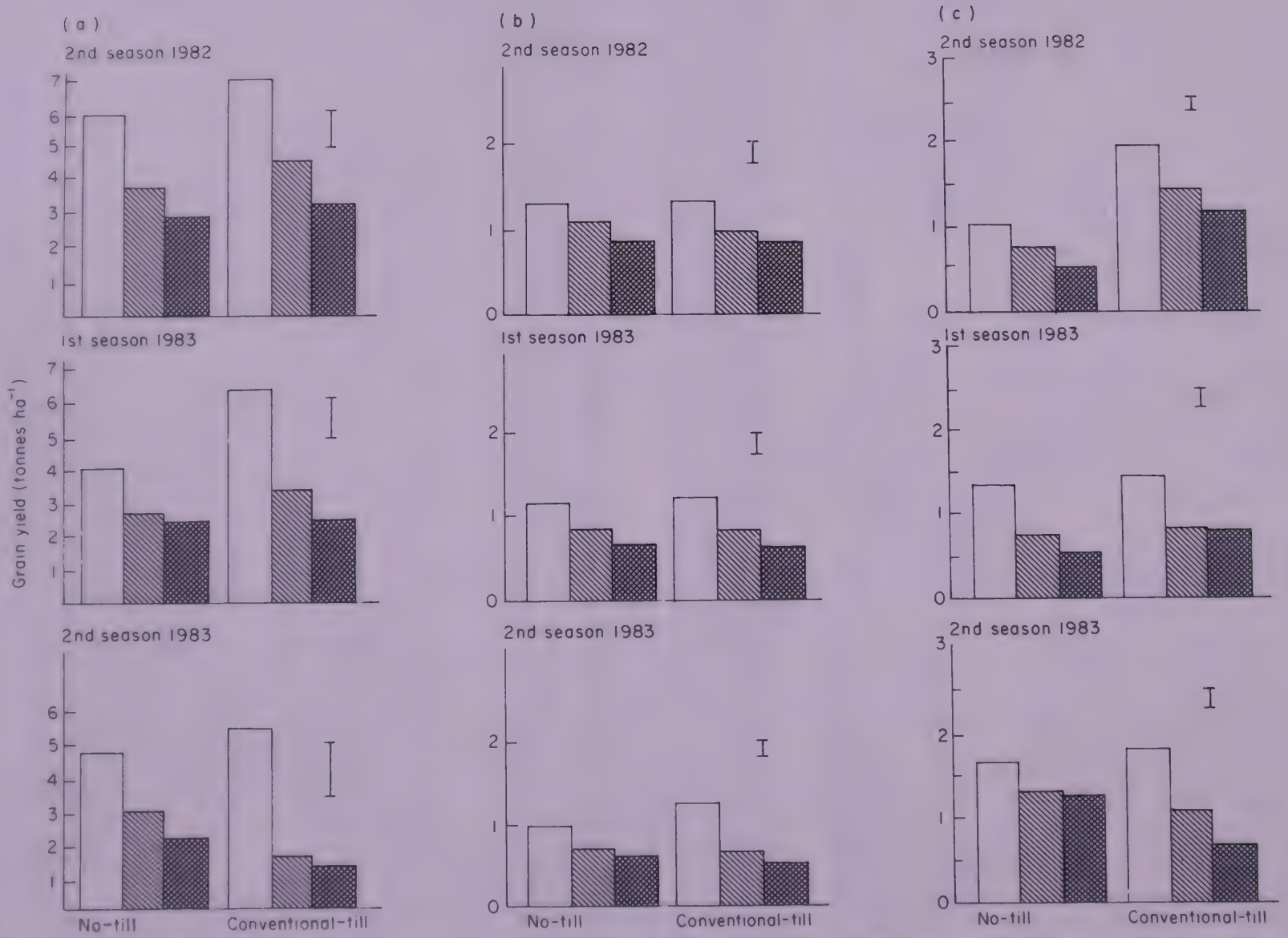

Figure 8. Grain yield of (a) maize, (b) cowpeas, and (c) soya beans for three consecutive seasons for different tillage and soil compaction treatments: (□), no-pass; (▨), two-pass; (▩), four-pass. l.s.d. 5%.

Table 3. Weight of maize grains per cob, weight of cowpeas grains per plant and unit weight per 100 grains of soya beans as affected by tillage and compaction treatments

Treatment		Mean values for three seasons (g)		
		Maize (weight of grains cob^{-1})	Cowpeas (weight of grains plant^{-1})	Soya beans (unit weight 100 grains^{-1})
00[a]	NT[b]	117.9b	18.2a	36.1a
02	NT	75.3c	14.3b	25.5b
04	NT	60.4d	11.6c	21.7bc
00	CT	156.3a	119.3a	32.4a
02	CT	79.7c	11.3c	20.9c
04	CT	58.8d	9.0d	14.9d

a–d: Letters denote significance at 5% level using Duncan's new multiple range test. Means with same letter are not significantly different.

[a] Number of passes of a 2-tonne roller.

[b] Tillage treatment: NT=no-tillage, CT=conventional tillage.

relating soil properties to grain yield are given in Table 5. Parameters in the equations are arranged in the order in which they were preferentially selected by the statistical procedure. In general, the coefficients of multiple correlations were more than the coefficient of simple correlation and ranged between 0.81 and 0.97. These results imply that in this study about 66 to 99% of variability in grain

Table 4. Relationship between soil physical properties and grain yield for the second season in 1983 crops

Soil property	Regression equation	Correlation coefficient (r)
Maize		
Dry bulk density (tonnes m^{-3})	$y= 17.34-8.84x$	0.80***
Total porosity (ratio)	$y=-6.33+23.74x$	0.79***
Equilibrium infiltration rate (cm h^{-1})	$y= 1.46+0.55x$	0.82***
Soil moisture (g g^{-1})	$y= 2.75+11.98x$	0.17 n.s.
Penetrometer resistance (MPa)	$y= 7.80-1.68x$	0.59*
Relative compaction (ratio)	$y= 17.64-17.34x$	0.80***
Specific volume (ratio)	$y= 8.50+6.64x$	0.75***
Cowpeas		
Dry bulk density (tonnes m^{-3})	$y= 3.10-1.43x$	0.73***
Total porosity (ratio)	$y=-0.69+3.76x$	0.72***
Equilibrium infiltration rate (cm h^{-1})	$y= 0.57+0.02x$	0.67**
Soil moisture (g g^{-1})	$y= 0.81+1.39x$	0.12 n.s.
Penetrometer resistance (MPa)	$y= 1.47-0.22x$	0.47*
Relative compaction (ratio)	$y= 3.12-2.77x$	0.72***
Specific volume (ratio)	$y=-1.12+1.09x$	0.69**
Soya beans		
Dry bulk density (tonnes m^{-3})	$y= 4.22-2.03x$	0.74***
Total porosity (ratio)	$y=-1.18+5.37x$	0.72***
Equilibrium infiltration rate (cm h^{-1})	$y= 0.47+0.04x$	0.88***
Soil moisture (g g^{-1})	$y= 0.85+2.89x$	0.13 n.s.
Penetrometer resistance (MPa)	$y= 2.14-0.46x$	0.58*
Relative compaction (ratio)	$y= 4.29-3.98x$	0.74***
Specific volume (ratio)	$y=-1.66+1.50x$	0.67**

y=Grain yield (tonnes ha^{-1}).

x=Soil property, measured at 100-mm depth, except equilibrium infiltration rate.

n.s., *, **, ***=not-significant, significant at 5, 1, 0.1% respectively.

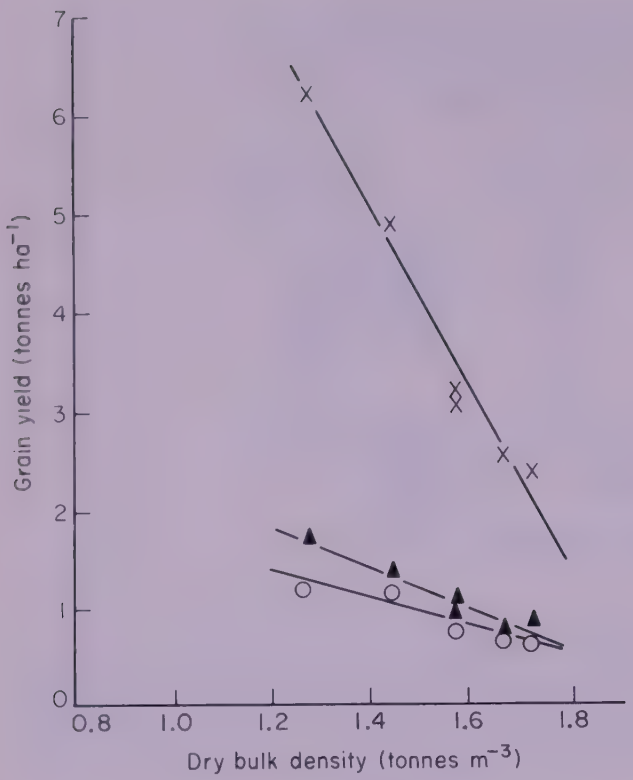

Figure 9. Comparative effects of soil bulk density on grain yields of: (×), maize—$y=17.93-9.22x$ ($r=-0.987^{***}$); (▲), soya bean—$y=4.41+2.15x$ ($r=-0.966^{**}$) and (○), cowpea—$y=3.12-1.44x$ ($r=-0.976^{***}$).

Table 5. Regression equations relating crop yield to soil properties

Crop	Regression equation	R
(a) Second season 1982:		
Maize	$y=8.80+0.1\ i+25.3\theta-37.0d^2-2.5e^2+14.9\varrho$	0.89***
Cowpeas	$y=71.4-4.5.2e^2+25.9\theta^2-0.01i+12.5d^2-254.6l\ -0.2P+77.6\varrho^2+153.2e$	0.83**
Soya beans	$y=-9.5+0.1i+2.4e-0.001i^2+2.0l^2+13.2\theta^2$	0.96***
(b) First season 1983:		
Maize	$y=-20.1+346.7e-0.02i^2-17.1\varrho-908.9d-83.8e^2+0.4P^2+654.7d^2-56.9\theta^2+0.6i$	0.97***
Cowpeas	$y=65.8-0.03i+19.5\theta-0.06P^2-73.4\theta^2-8.7e^2+24.5e-185.3d+105.1d^2$	0.81**
Soya beans	$y=1.6+0.001i^2-12.1\theta+50.0\theta^2-0.2e^2-0.2\varrho^2+0.2P$	0.96***
(c) Second season 1983:		
Maize	$y=-64.9-54.5\varrho^2-0.05i+27.2^2+0.01i^2-97.9e+184.3\varrho-0.2P+62.0\ \theta^2$	0.90***
Cowpeas	$y=3.3+0.5\varrho+0.03i+12e^2-4.1e-0.3i^2-0.04P$	0.90***
Soya beans	$y=-3.3-3.5\varrho^2+17.4d+0.2P-0.9P+32.2\theta^2$	0.92***

y=crop yield (t ha^{-1}), d=relative compaction (a ratio), e=specific volume (a ratio), i=equilibrium infiltration rate (cm h^{-1}), P=penetrometer resistance (MPa), θ=gravimetric water content (g g^{-1}), ϱ=dry bulk density (tonnes m^{-3}).

=Significant at 1% level, *=significant at 0.1% level.

yield of these crops was attributed to bulk density, infiltration rate, penetrometer resistance, relative compaction and specific volume.

In the second season 1982 and first season 1983, equilibrium infiltration rate was the most important soil physical property that was related to the yield of grain crops. With repeated compaction in the third consecutive cropping season (i.e. the second season of 1983), the dry bulk density was the soil physical property most highly correlated with grain yield, followed closely by the equilibrium infiltration rate.

4. Conclusion

The field experiment reported in this study leads to the following conclusions. (a) Four passes of the roller reduced percentage emergence, plant height, leaf area index, and dry matter yield of maize,

cowpea and soya bean. The reduction was more marked on ploughed than on no-till plots. (b) the two- and four-pass compaction treatments significantly reduced root growth of maize, cowpea and soya bean in the 0–70 mm depth. At each level of compaction, the root density of grain crops at all depths was greater for no-tillage than for ploughing. (c) Both two- and four-pass compaction treatments reduced leaf nutrient concentration in grain crops only in the third consecutive cropping season. (d) Both two- and four-pass compaction significantly decreased the grain yield of maize, cowpea and soya bean. Yield reduction was, however, severe in the heavily compacted treatment. The severity of the effect of compaction on grain yields of maize, cowpea and soya bean also increased with repeated compaction in subsequent seasons, and was particularly marked on ploughed plots. Traffic-induced compaction reduced grain yield more significantly in maize than in cowpea and soya bean.

The soil physical properties that correlated with grain yields were equilibrium infiltration rate, dry soil bulk density, total porosity, penetrometer resistance, relative compaction and specific volume. Grain yields were negatively correlated with bulk density, total porosity, and penetrometer resistance, and positively correlated with total porosity, infiltration rate and specific volume.

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J. Sci. Food Agric. 1986, **37**, 1155–1164

Extractability and Bioavailability of Zinc, Nickel, Cadmium and Copper in Three Danish Soils Sampled 5 Years After Application of Sewage Sludge

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Soils from three Danish experiments testing identical quantities of sewage sludge were sampled 5 years after the application of sludge ceased. Chemical studies on the soils involving single and sequential extractions, displaced solution measurements and plant uptake experiments in a glasshouse showed that sludge-added Zn, Ni, Cd and Cu persisted in extractable and bioavailable forms in the topsoils and that soil pH and texture influenced their chemistry and availability. The EDTA-extractabilities of native and of sludge-added Cd were similar, but native Zn, Ni and Cu were less extractable than sludge-added metals. 0.1 M-calcium chloride was the best extractant for predicting plant uptake. In some cases a chelating extractant combined with pH measurements is suitable if a single reagent is to be used for all four metals.

Keywords: Zinc; nickel; cadmium; copper; barley; beet; sewage sludge; chemical extractants.

1. Introduction

Sewage sludge containing high concentrations of potentially toxic elements such as Zn, Ni, Cd and Cu poses problems when disposed of on agricultural land. Added metals may accumulate and persist in soils and cause enhanced plant uptake many years after sludge applications have ceased.[1] Metals may also reduce the size and activity of the soil microbial biomass.[2,3]

The chemistry and bioavailability of added metals have been studied frequently, but over relatively short periods following sludge application; long term effects have rarely been measured. However, such information is indispensable to set criteria for applying sludge to agricultural soils.

During 1973–1979 sewage sludge originating from two sewage treatment plants, one in a rural and one in an industrial area, was added to soils on three experimental sites, Askov, Lundgaard and Rondhave, in Denmark. In each experiment, comparable treatments received almost identical quantities of metals. The experimental design, crop yields, crop and sludge composition, and results of some soil analyses have been given.[4,5]

The fate of metals added to these soils can now be studied without complications due to rapidly decomposing sludge. The soils also give an opportunity to compare the effectiveness of several chemical extractants commonly used for predicting metal uptake by crops.

2. Experimental

2.1. Soil treatments

The three experimental sites are situated in South Jutland (55°N, 9°E) with similar annual precipitation, 750 mm, and mean temperature, 8°C. The soil at Lundgaard (4% clay, 5% silt, 2.3% o.m.) is a

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coarse sand, while those at Askov and Rondhave are both sandy loams with 11% clay, 12% silt, 2.7% o.m. and 15% clay, 20% silt and 2.6% o.m. respectively.

The treatments[4] sampled for the present study were: no. 2 (6 t ha^{-1} $year^{-1}$ of sand-free sludge dry matter with low-metal content), no. 3 (same dressing as no. 2, but high-metal content), no. 4 (no sludge added, but N and P given in fertilisers), no. 5 (three times the sludge dressing of no. 2), and no. 6 (three times the sludge dressing of no. 3). The sludge was added during the period 1973–1978 and the total amounts of Zn, Ni, Cd and Cu added are given in Table 1. The sludge with high-metal content was heavily limed, increasing soil pH. Various horticultural and agricultural crops were grown in rotation before sludge additions ceased, and then there was a rotation of grass, wheat, beetroots and spring barley.

Table 1. Amounts of metals (kg ha^{-1}) added in sludge to the soils during 1973–1978

Treatment	Number	Zn	Ni	Cd	Cu
No sludge	4	0	0	0	0
1S-LM	2	97	3	0.5	17
1S-HM	3	117	13	1.2	55
3S-LM	5	290	9	1.4	50
3S-HM	6	350	40	3.5	165

1S=6 t sludge dry solids ha^{-1} y^{-1}; 3S=18 t; LM=low metal sludge; HM=high metal sludge.

A composite sample of 20 cores (approximately 4 kg) of topsoil (0–20 cm) was collected in January 1984 from each of the five treatments on each of the three sites. The soil was air-dried, crushed to pass a 2 mm mesh and then sent to Rothamsted.

Total metal contents were determined by extracting the soils with hot aqua regia (100°C).[6] Metals extracted with 0.05 M EDTA at pH 7,[7] with 0.005 M DTPA at pH 7.3[8] and with 0.1 M $CaCl_2$ overnight[9] (with shaking at 120 strokes min^{-1}) were also determined; all analyses were in triplicate.

2.2. Extraction into calcium chloride at different pH values

Twelve 5 g samples of each soil treatment were shaken overnight with 0.01 M $CaCl_2$, (not 0.1 M as above) the pH being adjusted to a range of values between 4 and 7 using dilute HCl or NaOH. The mixtures were centrifuged and Zn, Ni, Cd and Cu determined in the centrifugates. Additionally, for solutions extracted from Lundgaard soils only, soluble organic matter was determined by dichromate oxidation and Zn^{2+}, Ni^{2+}, Cd^{2+} and Cu^{2+} were determined by an ion exchange equilibration method.[10]

2.3. Sequential extractions

The sequential extraction procedure has been described in detail;[11] briefly, it consisted of extracting the soil by four reagents in turn. In the first stage duplicate 5 g samples of each soil were shaken overnight with 0.01 M $CaCl_2$; in the second stage the residue was treated overnight with unbuffered 10 volumes H_2O_2 before heating to 60°C for 3 h, and in the third stage the residue was shaken overnight with 25% acetic acid (HOAc). The final residue was then digested with aqua regia. After each stage the mixture was centrifuged and the residue washed with water. Zn, Ni, Cd and Cu were determined in the centrifugates.

2.4. Pot experiments

One kilogram (three times) of each soil was kept in pots at 90% water holding capacity (WHC) for about 6 weeks and 40 ml of the soil solution was then displaced from each pot using deionised water. The solution was centrifuged and Zn, Cd, Ni and Cu were determined in the centrifugate. Barley (cv. Georgie) and red beet (cv. Red globe) were grown in the pots successively for 6 and 7 weeks respectively; they were given a nutrient solution containing NH_4NO_3 (3.8 g litre $^{-1}$), K_2HPO_4 (3.7 g

litre $^{-1}$) and $MgSO_4$ (0.03 g litre $^{-1}$) periodically. The barley shoots and beet leaves were harvested, air-dried, weighed and ground in a stainless steel mill, and then analysed after digestion in nitric acid.

2.5. Analytical methods

Zn, Ni, Cd and Cu together with P, K, Ca, Mg, Na, S, Fe, Mn, Al, Ti, Cr and Pb were initially determined in all soil and plant digests and extracts using an ARL 34000 inductively coupled plasma optical emission spectrometer (i.c.p.). When Zn, Ni, Cd or Cu concentrations in solutions were near or below the detection limits for each element, analyses were repeated for the relevant element using a Perkin-Elmer 372 atomic absorption instrument with a HGA-2200 attachment (flameless a.a.s.).

3. Results and discussion—soils

3.1. Total metal contents

The total concentrations of some metals in, and the pH of, the 15 soils are given in Table 2. The ratios of metals added in high-metal to low-metal sludges were 1.2 (Zn), 4.3 (Ni), 2.4 (Cd) and 3.2 (Cu). These differences in metal composition gave different total metal concentrations in the different treatments in which the sludges were added to soil. Concentrations in non-amended soils agree with background levels of Danish arable soils.[12]

Table 2. pH value of, and total metal concentrations (percentage Fe; mg kg^{-1} others) in each soil

Soil	Treatment	pH[a]	Fe	Mn	Zn	Ni	Cd	Cu
Askov	No sludge	5.75	0.75	173.3	27.5	4.47	0.23	6.7
	1S-LM	5.65	0.76	201.2	50.6	5.70	0.33	10.8
	1S-HM	6.09	0.77	172.8	55.8	7.49	0.73	20.5
	3S-LM	5.77	0.79	205.0	81.2	5.79	0.71	16.9
	3S-HM	5.88	0.79	177.0	107.6	12.36	1.74	42.6
Lundgaard	No sludge	4.55	0.32	213.8	14.7	1.00	0.13	4.0
	1S-LM	4.65	0.36	229.6	30.2	1.98	0.22	6.3
	1S-HM	5.10	0.35	211.9	35.1	3.71	0.29	13.5
	3S-LM	4.61	0.45	309.4	58.4	3.14	0.39	13.9
	3S-HM	5.87	0.42	295.3	82.9	9.37	0.80	36.1
Rondhave	No sludge	5.75	1.16	226.0	42.7	8.00	0.38	9.9
	1S-LM	5.75	1.29	237.4	63.8	9.58	0.53	13.8
	1S-HM	5.85	1.27	245.7	67.4	11.12	0.80	21.6
	3S-LM	5.72	1.18	231.2	105.9	9.89	0.80	21.2
	3S-HM	5.84	1.15	232.3	125.1	16.09	2.08	47.0

[a]In 1/2.5 soil: 0.01 M $CaCl_2$.
For treatment numbers see Table 1.

3.2. Extractable metal contents

EDTA, DTPA and $CaCl_2$ each extracted a higher proportion of the total metal content from sludge-treated soils than from the control soil from the same site, despite the fact that the soils were sampled 5 years after the last application of sludge. Similarly, proportions of total metal were higher in solutions displaced from sludge-amended soils than from the control soil from the same site. *Metal concentrations in individual extracted solutions and displaced soil solutions are available on request.*

The percentage of Zn extracted by EDTA from all Lundgaard soil-sludge treatments and from high-metal sludge treatments of the other soils was comparable with that found by McGrath in his studies on a sandy loam amended with sludge.[1] The percentage of Ni was in general somewhat smaller and cadmium somewhat larger than those found by McGrath, while the copper data generally accord with his.

3.3. Extraction at different pH values

The pH of the soils from each treatment varied on each site (Table 2). It is not possible to compare the effect of different metal loadings and soil textures on metal extractabilities, particularly by

Table 3. Percentages of total Zn extracted into the solution phase after shaking soils with 0.01 M $CaCl_2$ at various pH values

Soil	Treatment	Percentage of metal in solution at				
		pH 4.5	pH 5.0	pH 5.5	pH 6.0	pH 6.5
Askov	No sludge	2.0	1.0	0.4	0.1	0.05
	1S-LM	8.4	4.7	2.3	0.8	0.05
	1S-HM	10.1	5.5	2.6	0.8	0.08
	3S-LM	13.6	8.0	3.4	0.9	0.1
	3S-HM	13.9	7.6	3.7	1.1	0.1
Lundgaard	No sludge	14.3	8.8	4.6	1.5	0.3
	1S-LM	23.6	16.0	7.9	2.6	0.7
	1S-HM	20.0	11.2	6.0	2.6	0.5
	3S-LM	23.6	16.0	8.0	3.4	0.9
	3S-HM	21.9	11.2	5.4	2.6	0.8
Rondhave	No sludge	1.8	0.8	0.4	0.2	0.05
	1S-LM	6.4	3.4	1.4	0.5	0.1
	1S-HM	8.0	3.9	1.6	0.5	0.1
	3S-LM	12.6	6.6	2.8	1.0	0.3
	3S-HM	11.5	5.0	2.2	0.7	0.3

For treatment numbers see Table 1.

unbuffered reagents, unless measurements are carried out at the same soil pH. Extractions using $CaCl_2$ were therefore made at a range of pH values, which also gave an indication of the extractabilities of different metals at different pH values, and of the change in metal speciation in solution with pH. Tables 3–6 give the percentages of total metals in the solution phase at various pH values (obtained by interpolation of measured values—*actual concentrations in individual solutions are available on request*).

Zinc concentrations (Table 3) increased as pH decreased over the whole range tested, particularly at high metal loadings. At any pH the highest concentrations of Zn were found in extracts of the very coarsely textured Lundgaard soil. The percentage of total Zn brought into solution increased as the total metal loading of Zn increased. The same trends were observed for Ni (Table 4).

Cadmium concentrations (Table 5) also increased as pH decreased over the whole range tested. There was however no trend for sludge-added Cd to be more extractable than native Cd, and Cd concentrations in Rondhave soil extracts were higher than in Lundgaard soil extracts at the same pH.

Table 4. Percentages of total Ni extracted into the solution phase after shaking soils with 0.01 M $CaCl_2$ at various pH values

Soil	Treatment	Percentage of metal in solution at				
		pH 4.5	pH 5.0	pH 5.5	pH 6.0	pH 6.5
Askov	No sludge	1.6	0.9	0.6	0.6	0.6
	1S-LM	5.9	3.7	2.0	0.8	0.8
	1S-HM	6.9	3.8	2.2	1.0	0.8
	3S-LM	7.3	4.4	2.4	1.4	1.2
	3S-HM	11.0	6.6	4.0	1.8	1.0
Lundgaard	No sludge	6.8	4.2	2.3	1.2	0.9
	1S-LM	10.8	7.4	4.2	2.3	1.6
	1S-HM	15.9	10.4	6.2	3.4	2.0
	3S-LM	12.8	8.5	5.2	3.2	2.1
	3S-HM	20.8	12.6	7.0	4.2	2.3
Rondhave	No sludge	1.4	0.6	0.3	0.2	0.2
	1S-LM	2.1	0.9	0.5	0.2	0.2
	1S-HM	4.2	2.2	1.3	0.8	0.7
	3S-LM	4.5	2.5	1.4	0.8	0.7
	3S-HM	7.9	4.0	2.1	1.3	0.7

For treatment numbers see Table 1.

Table 5. Percentages of total Cd extracted into the solution phase after shaking soils with 0.01 M $CaCl_2$ at various pH values

Soil	Treatment	Percentage of metal in solution at				
		pH 4.5	pH 5.0	pH 5.5	pH 6.0	pH 6.5
Askov	No sludge	17.0	10.5	5.5	2.0	*
	1S-LM	15.5	9.5	5.5	2.0	*
	1S-HM	10.5	6.0	3.0	1.5	*
	3S-LM	9.5	5.0	2.5	1.0	*
	3S-HM	9.5	5.5	3.0	1.0	*
Lundgaard	No sludge	19.5	15.0	9.5	5.5	4.5
	1S-LM	19.0	15.5	10.0	5.0	3.0
	1S-HM	24.0	18.5	12.0	7.0	4.5
	3S-LM	18.0	14.0	9.5	8.5	3.5
	3S-HM	18.0	13.0	7.5	4.0	2.5
Rondhave	No sludge	14.0	9.0	5.0	2.5	1.0
	1S-LM	16.5	11.0	6.5	3.0	1.0
	1S-HM	15.0	10.0	6.0	3.0	1.0
	3S-LM	16.5	11.0	6.5	3.5	1.5
	3S-HM	10.0	6.0	3.5	2.0	1.0

For treatment numbers see Table 1.
* Not measured.

Table 6. Percentages of total Cu extracted into the solution phase after shaking soils with 0.01 M $CaCl_2$ at various pH values

Soil	Treatment	Percentage of metal in solution at				
		pH 4.5	pH 5.0	pH 5.5	pH 6.0	pH 6.5
Askov	No sludge	0.26	0.25	0.24	0.23	0.24
	1S-LM	0.18	0.15	0.14	0.16	0.24
	1S-HM	0.21	0.14	0.12	0.11	0.16
	3S-LM	0.28	0.22	0.18	0.19	0.30
	3S-HM	0.35	0.14	0.08	0.09	0.16
Lundgaard	No sludge	1.35	1.00	0.83	1.02	1.45
	1S-LM	0.65	0.50	0.45	0.50	0.65
	1S-HM	0.72	0.50	0.39	0.37	0.37
	3S-LM	0.56	0.42	0.40	0.44	0.52
	3S-HM	0.73	0.50	0.33	0.25	0.22
Rondhave	No sludge	0.52	0.47	0.43	0.40	0.39
	1S-LM	0.60	0.47	0.37	0.31	0.30
	1S-HM	0.98	0.70	0.52	0.43	0.38
	3S-LM	0.90	0.68	0.49	0.43	0.44
	3S-HM	0.95	0.67	0.47	0.32	0.30

For treatment numbers see Table 1.

In contrast, it was at pH values below 5 that copper concentrations fell most rapidly as pH rose (Table 6). In several cases they reached minima at pH 5.5–6 and then rose very slightly. Organic carbon concentrations in Lundgaard soil extracts (the only ones to be measured) also showed minima at pH 5.5–6, and then rose slightly, so presumably Cu was taken into solution as organic complexes.

Determinations of metal ion concentrations on solutions extracted from Lundgaard soils showed that Zn existed almost entirely (>90%) as Zn^{2+} in them at all pH values up to 7. Nickel existed almost entirely as Ni^{2+} below pH 6 but there was some complex formation above this pH giving about 80% of Ni^{2+} at pH 7. The percentage of Cd in these solutions was always ~70%; this percentage was not pH-dependent and it was calculated that the remainder was present as the $CdCl^+$ ion. In contrast, the proportion of Cu present as Cu^{2+} was about 40% at pH 4.5 and fell to <10% at pH 7, the remainder being presumably present as organic complexes.

The effects of pH and soil texture on the three metals Zn, Ni and Cu in this study are similar to those observed in three British soils to which sludge had recently been added[13] and to effects in a

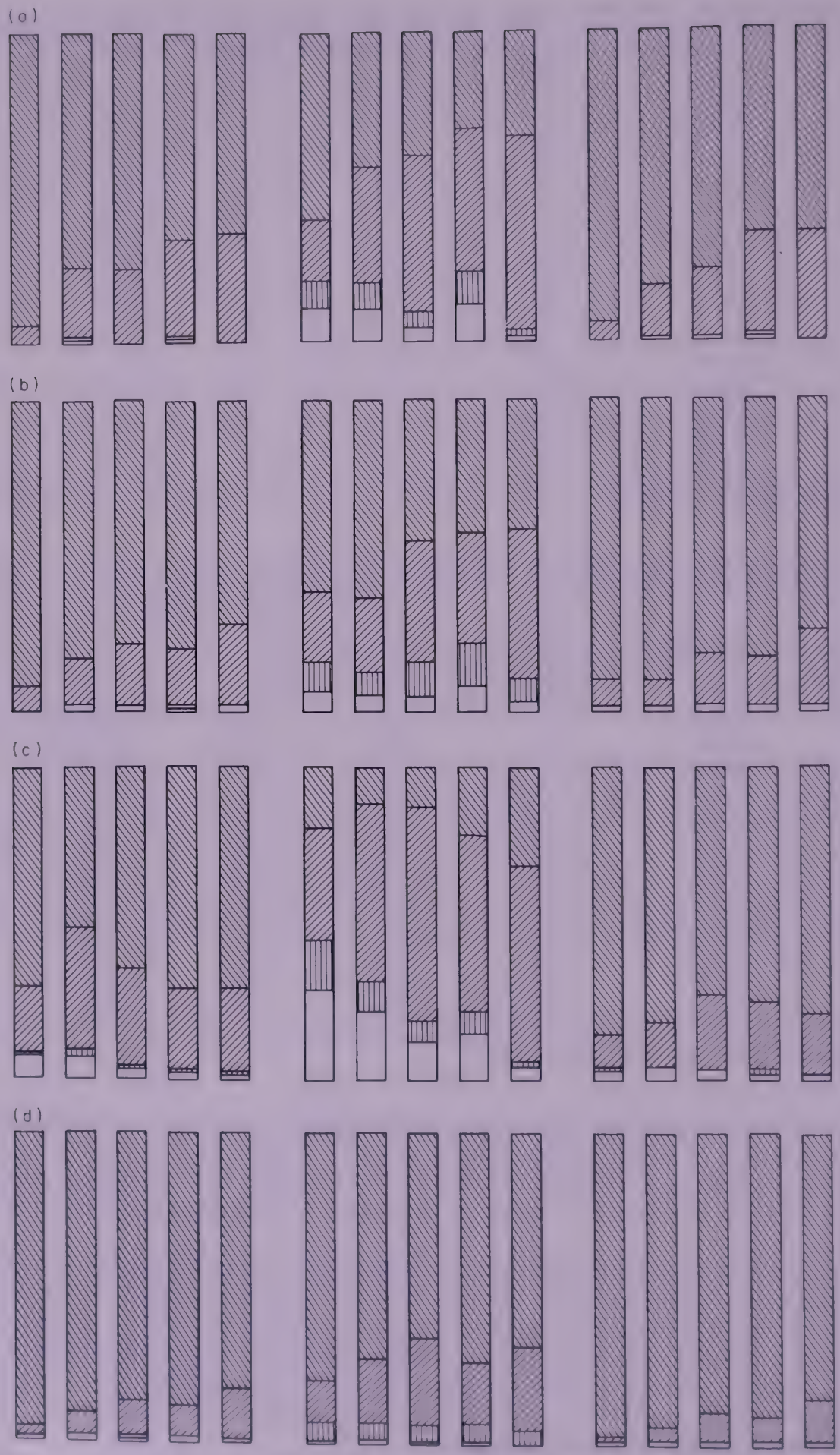

Figure 1. Percentages of total metals extracted sequentially from soils by (□), $CaCl_2$; (▥), H_2O_2; (▨), HOAc; and (▧), aqua regia. Soil and sludge treatment orders as in Tables 3–6. (a) Zn, (b) Ni, (c) Cd, (d) Cu.

series of Belgian soils.[14] In the British and Belgian work the soils used had a wide range of textures, and both soil pH and texture had a marked effect on Zn and Ni concentrations, but not on Cu. In the present study soil texture had only a small effect because all three soils were sands or sandy loams.

3.4. Sequential extraction

Sequential extraction schemes have been used to try to divide the total quantity of a trace element in soil into different pools according to their solid state forms.[15] They have also been used in connection with plant growth experiments[16] to divide elements into 'functional' pools of immediately available, potentially plant-available and unavailable. The scheme followed here aimed to extract successively exchangeable, organically bound, oxide bound and residual metals, and to assess the relationship of such pools to bioavailability using correlation analysis.

The results for Zn, Ni, Cd and Cu are presented as percentages of the total metal extracted at all four stages (Figure 1, a–d). This total, as found by others,[17] did not equal the total found in the soil by a single aqua-regia extraction (Table 2), but usually came within 20% of it. Differences between individual soils, sludge treatments or metals would appear the same if the results were presented as percentages of the total found by a single extraction, rather than as percentages of the total metal extracted sequentially.

The concentrations of individual metals extracted by $CaCl_2$ were influenced greatly by the initial pH of the soil but were noticeably smaller for Cu than other metals. The ratio of H_2O_2-extractable metal to $CaCl_2$-extractable metal shows the relative importance of organic matter binding for the various elements—this is most marked for Cu but there is a tendency for it to be important for Ni.

As a percentage of the total of metals extracted, that by the first three stages was nearly always largest in the sandy Lundgaard soil and among treatments was always smallest for the no-sludge treatment. This happened even for Cu, suggesting that after organic matter had been partly removed by H_2O_2, the added Cu associated with it was more extractable than native Cu. The quantities removed by the end of the acetic acid extraction stage were comparable to those removed by a single extraction with $CaCl_2$ at the lowest pH used, about pH 3.

4. Results and discussion—plants

4.1. Plant yields

There was no effect either of soil or of sludge treatment on the yield of barley plants. Red beet plants grown on Lundgaard soil were considerably smaller than those grown on the other two soils and on treatments 2, 4 and 5 failed presumably because of acidity rather than metal toxicity (Table 2). There were no effects attributable to high metal concentrations on either crop.

4.2. Plant metal concentrations

4.2.1. Introduction

Zinc, nickel and cadmium concentrations in both crops were higher in plants grown on Lundgaard soil than on the other two soils, even where the pH values of the treatments were approximately the same. In the case of beet plants, however, the small size of the plants may have contributed to this. Copper concentrations were approximately the same in all the plants. *All metal concentrations in plant dry matter are available on request.*

4.2.2. Correlations—single extractants

Table 7 shows values of correlation coefficients (r^2) between metal concentrations in plant tissue dry matter and total metals and EDTA-, DTPA-, $CaCl_2$-extractable and soil solution metal concentrations, and also coefficients of multiple regression between plant metal concentrations and these five metal concentrations + soil pH, over all soils. *Regression coefficients for individual soils are available on request.* The correlations were made using triplicate measurements, i.e. there were 15 data sets for each soil and 45 for the three soils altogether for barley. Because the beet failed on three treatments there were 36 sets for beet. All data except for pH were log-transformed before

correlation analysis, because the presence of a small number of high-metal results gave skewed distributions of untransformed data.

The results for Zn, Ni and Cd can be summarised thus: (a) r^2 values in the exclusion of soil pH became higher as smaller amounts of metals were extracted (i.e. $CaCl_2$ extracts and displaced solutions). This was of course also observed during sequential extraction and reinforces the view that the exchangeable fraction approximates to the bioavailable fraction of these metals. (b) In many cases highly significant values of r^2 were obtained when data from any one soil was used, but they became very small when measurements from all soils were used. This happened most often when the proportions of the metals extracted were large. (c) When the soil pH was added to the correlation, r^2 values involving chelating buffered extractants often became noticeably higher, but this did not apply for the unbuffered $CaCl_2$ (except for Cd).

Table 7. Squared correlation coefficients between metal concentrations in plant tissue dry matter Mp and metal concentrations in each extractant M_s, and multiple correlation coefficients between M_p and M_s+soil pH, over all soils

			r^2 for each extractant				
Metal	Plant	Correlation	Total	EDTA extract	DTPA extract	$CaCl_2$ extract	Displaced solution
Zn	Barley	M_p *vs* M_s	0.03	0.32***	0.48***	0.78***	0.60***
		M_p *vs* M_s+pH	0.55***	0.72***	0.74***	0.80***	0.61***
	Beet	M_p *vs* M_s	0.29***	0.62***	0.74***	0.82***	0.49***
		M_p *vs* M_s+pH	0.57***	0.79***	0.85***	0.85***	0.50***
Ni	Barley	M_p *vs* M_s	0.00	0.10	0.17**	0.41***	0.24***
		M_p *vs* M_s+pH	0.31***	0.46***	0.50***	0.47***	0.37***
	Beet	M_p *vs* M_s	0.03	0.51***	0.64***	0.80***	0.34***
		M_p *vs* M_s+pH	0.09	0.65***	0.74***	0.81***	0.37***
Cd	Barley	M_p *vs* M_s	0.07	0.12	0.09	0.11	0.07
		M_p *vs* M_s+pH	0.08	0.14	0.10	0.12	0.15**
	Beet	M_p *vs* M_s	0.00	0.01	0.02	0.22**	0.15
		M_p *vs* M_s+pH	0.24**	0.30***	0.33***	0.41***	0.31***
Cu	Barley	M_p *vs* M_s	0.57***	0.60***	0.66***	0.54***	0.32***
		M_p *vs* M_s+pH	0.61***	0.64***	0.67***	0.59***	0.46***
	Beet	M_p *vs* M_s	0.43***	0.47***	0.43***	0.27***	0.16
		M_p *vs* M_s+pH	0.43***	0.48***	0.43***	0.27***	0.17**

**$P<0.01$.
***$P<0.001$.

None of the above trends occurred with Cu. There was a slight increase in magnitude of r^2 values as the proportion extracted became larger, and little advantage in including pH in the correlations. These results agree with those of Tills and Alloway[18] who found that the highest correlation coefficients between Cu concentrations in barley and in solutions extracted from a range of soils came when they used 0.05 M EDTA as their extractant.

On the basis of correlation coefficients 0.1 M $CaCl_2$ is the 'best' extractant for Zn, Cd and Ni among those tried. Sauerbeck and Styperek[9] considered it to be the best extractant for Zn and Cd in a study involving 10 soils and a large range of crops. However, for every element there is a certain concentration below which it becomes difficult to determine analytically because of lack of instrumental sensitivity, contamination problems and possible matrix and major element interferences. In this work this concentration was about 0.02 mg litre^{-1} for Zn, Cd and Cu and 0.1 mg litre^{-1} for Ni. Above this concentration elements were determined using simultaneous i.c.p. spectrometry (which is more sensitive than flame a.a.s.) but below it use of flameless a.a.s. was necessary. Only Zn of the four elements studied could be determined in $CaCl_2$ extracts using i.c.p. but all four could be so determined in DTPA and EDTA extracts. On this basis DTPA is to be preferred, particularly if multiple correlations including soil pH are used. The problem with measuring metal concentrations in $CaCl_2$ extracts applies still more to displaced solutions with even smaller concentrations.

Table 8. Single correlation coefficients (r) between metal concentrations in plant dry matter and cumulative quantities of metals extracted sequentially by $CaCl_2$, H_2O_2, HOAc and aqua regia, taken over all soils

		r values for metals extracted by			
Metal	Plant	Stage 1	Stages 1+2	Stages 1+2+3	Stages 1+2+3+4
Zn	Barley	0.90***	0.92***	0.77***	0.22
	Beet	0.92***	0.93***	0.88***	0.59
Ni	Barley	0.71**	0.76***	0.57	0.08
	Beet	0.97***	0.97***	0.90***	0.33
Cd	Barley	0.31	0.22	0.57	0.29
	Beet	0.76***	0.87***	0.40	−0.28
Cu	Barley	0.65**	0.65***	0.79***	0.88***
	Beet	0.48	0.58	0.67**	0.80**

**$P<0.01$.
***$P<0.001$.

4.2.3. Correlations—sequential extraction

Table 8 gives single correlation coefficients between metal concentrations in barley and beet tops and cumulative quantities of metals extracted after each stage of the sequential extraction scheme. The data were log-transformed before correlation analysis, as with the data on single extractants.

For Zn, Ni and Cd the highest correlation coefficients occurred using the weak extractants $CaCl_2$ and H_2O_2, which again suggests that the relatively small exchangeable fraction of these metals is the most bioavailable. Similar conclusions were reached by LeClaire *et al.*[16] for Zn, though their scheme was rather different. Such a fraction would be susceptible to rapid accumulation and depletion as the pH changed, and this should be reflected in plant concentrations.

In the case of Cu however the highest correlation coefficients occurred between concentration in plant tops and the sum of all extractions. Though the possibility that there is a small fraction of Cu forming a bioavailable pool cannot be excluded, this particular extraction scheme does not identify one and suggests that it does not include organically complexed or exchangeable Cu.

5. Conclusions

The general conclusions to be drawn from this study are: (a) Five years after the last application of sludge Zn, Ni, Cd and Cu persisted in the topsoil in forms available to plants and extractable by chelating reagents and neutral salt solutions. (b) Zn, Cd and Ni possessed relatively small bioavailable fractions which approximated to exchangeable metal, and their size was very susceptible to pH changes. (c) Metals added to the light soil from Lundgaard were very much more extractable and bioavailable than those added to the other soils if the effect of pH is removed. (d) Cu behaves differently to Zn, Ni and Cd because of its high affinity for soil organic matter, which is shown most clearly by soil solution measurements. (e) 0.1 M $CaCl_2$ is the best extractant among those tried for predicting plant concentrations, but a combination of pH measurements and DTPA extraction is recommended for routine use, because of the difficulty of measuring small concentrations in $CaCl_2$ extracts.

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Log-linear Response Functions and Their Use to Model Data from Plant Nutrition Experiments

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Inverse polynomial response surfaces have been very useful as models for data when responses are not symmetrical about their optimum value or when they approach an asymptote. But a special computing routine is required, and the parameter estimates do not have exactly known distributions. An alternative family of models having the same flexibility of shape is the family of log-linear response functions, which may be fitted using standard multiple regression routines. They provide parameter estimates with very good statistical properties and have been used to explain several sets of data. Examples are given of their fit to data from an experiment on the nutrition of black currants grown under controlled conditions. From contour diagrams, it is easy to propose the best levels of fertilisers to be applied so as to obtain maximum crop and healthy growth.

Keywords: Response surfaces; inverse polynomials; log-linear models; multiple regression; plant nutrition.

1. Possible types of response function

In an experiment, the *response* y may depend on k independent, or predictor, quantitative variables. To define this relationship, let $\mathbf{x}=[x_1, x_2, \ldots, x_k]$ be a vector of predictor variables and $\boldsymbol{\beta}=[\beta_1, \beta_2, \ldots, \beta_h]$ be a vector of h unknown parameters: then

$$y=f(\mathbf{x}, \boldsymbol{\beta}) \tag{1.1}$$

where f is the *response function*.

Mead and Pike[1] discussed a number of response functions that have been widely used in biological science. Often the analysis of a set of data aims to find a set of values of $[x_1, x_2, \ldots, x_k]$ which provide the optimum value of y. Thus in an experiment using a single independent variable x, the simplest curved relationship

$$Y \equiv \mathrm{E}[y]=\beta_0+\beta_1 x+\beta_2 x^2$$

has its optimum (the maximum value of Y) at $x=-\beta_1/(2\beta_2)$. The parameters $[\beta_0, \beta_1, \beta_2]$ and their standard errors can be estimated simply.

When an experiment contains many independent variables (factors) $[x_1, x_2, \ldots, x_k]$, a general second-order model is

$$Y=\beta_0+\sum_{i=1}^{k}\beta_i x_i+\sum_{i=1}^{k}\beta_{ii}x_i^2+\sum_{\substack{i=1\\ i<j}}^{k}\sum_{j=1}^{k}\beta_{ij}x_i x_j \tag{1.2}$$

These *ordinary polynomials* are widely used in industrial statistics, but may be objected to for

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biological use on various grounds: (a) this family of functions does not include one with a plateau or asymptotic form of relationship, which is often found in biometry; (b) biological data rarely show a relationship that is symmetric about the optimum. Nelder[2] introduced *inverse polynomial* response surfaces to meet these objections. These take the general form

$$\prod_{i=1}^{k} x_i/y = P[x_1, x_2, \ldots, x_k] \tag{1.3}$$

where P is a polynomial and y is the response. Nelder[2] and Pike[3] advocated these surfaces as giving responses that are non-negative and bounded for all $\mathbf{x}$. However, this shape of response only arises when the parameters $\boldsymbol{\beta}$ in P are constrained to be positive. In estimation when constraints are placed on the parameters, the assumption of a normal error distribution no longer leads to normally distributed estimates $\{\beta_i\}$; and the residual sum of squares no longer follows the standard χ^2 distribution. Also, experience of examining many sets of data leads us not to expect all $\{\beta_i\}$ to be positive. We thus prefer unconstrained methods of estimation in fitting models to experimental results.

2. Possible shapes of inverse polynomial

A response function of the form

$$x/y = P_l(x) \tag{2.1}$$

with P_l a polynomial of order l in the single variable x will be called the inverse lth order polynomial; important special cases are

$$x/y = \beta_0 + \beta_1 x \tag{2.2}$$

the inverse linear relation, which may be rewritten

$$y^{-1} = \beta_0/x + \beta_1 \tag{2.3}$$

and

$$y^{-1} = \beta_0/x + \beta_1 + \beta_2 x \tag{2.4}$$

which is an inverse quadratic relation. Further terms, such as $\beta_3 x^2$, may be added to equation 2.4 but do not alter the shape of response fundamentally from the quadratic. Figures 1a–d show a range of shapes of $y^{-1} = \beta_0/x + \beta_1 + \beta_2 x + \beta_3 x^2$: in (a), $\beta_2 = \beta_3 = 0$ and in (b) $\beta_3 = 0$.

3. Extension to two factors, x_1 and x_2

The two-factor linear inverse polynomial is

$$x_1 x_2/y = \beta_{00} + \beta_{10} x_1 + \beta_{01} x_2 + \beta_{11} x_1 x_2 \tag{3.1}$$

which may be written

$$y^{-1} = \frac{\beta_{00}}{x_1 x_2} + \frac{\beta_{10}}{x_2} + \frac{\beta_{01}}{x_1} + \beta_{11} \tag{3.2}$$

showing that y^{-1} is bilinearly related to x_1^{-1} and x_2^{-1}. In the same way, the full two-factor quadratic model is

$$x_1 x_2/y = \beta_{00} + \beta_{10} x_1 + \beta_{01} x_2 + \beta_{11} x_1 x_2 + \beta_{20} x_1^2 + \beta_{02} x_2^2 + \beta_{21} x_1^2 x_2 + \beta_{12} x_1 x_2^2 + \beta_{22} x_1^2 x_2^2 \tag{3.3}$$

and on dividing through by $x_1 x_2$ a form analogous to (3.2) may be found. If $\beta_{00} = 0$ in (3.2), we have the

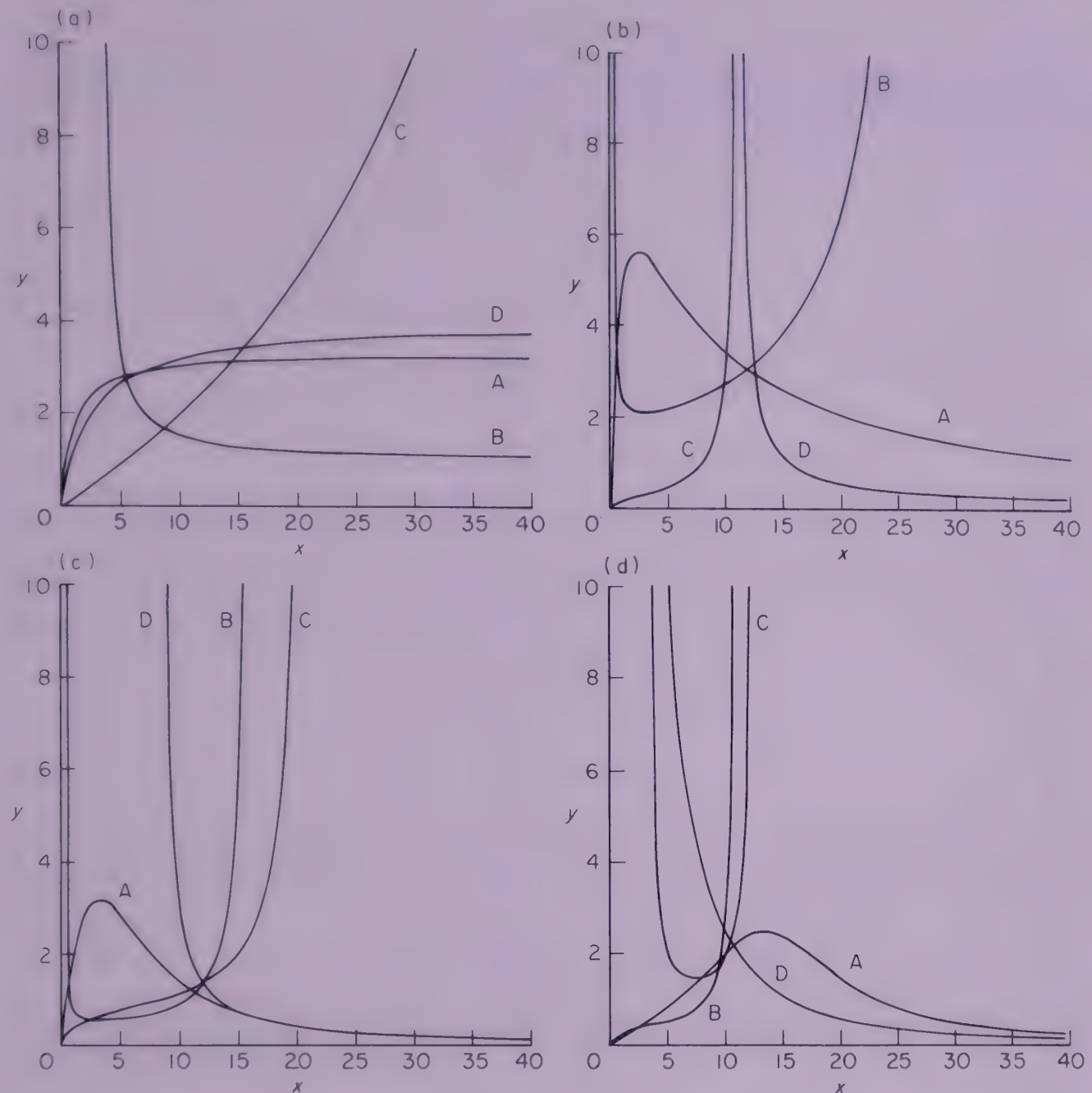

Figure 1. Graphs of the function $y^{-1}=\beta_0/x+\beta_1+\beta_2 x+\beta_3 x^2$. (a) $\beta_2=\beta_3=0$. A: $(\beta_0, \beta_1)=(0.3, 0.3)$. B: $(\beta_0, \beta_1)=(-3.5, 1)$. C: $(\beta_0, \beta_1)=(6, -0.1)$. D: $(\beta_0, \beta_1)=(0.64, 0.25)$. (b) $\beta_3=0$. $(\beta_0, \beta_1, \beta_2)=(0.12, 0.08, 0.02)$ in A; $(-0.17, 0.6, -0.022)$ in B; $(5, 2.5, -0.27)$ in C; $(-21.3, 0.8, 0.1)$ in D. (c) $(\beta_0, \beta_1, \beta_2, \beta_3)=(0.45, 0.1, 0.007, 0.005)$ in A; $(-1.05, 2.4, -0.07, -0.005)$ in B; $(1, 1.4, -0.05, -0.001)$ in C; $(-10, 0.7, 0.013, 0.005)$ in D. (d) $(\beta_0, \beta_1, \beta_2, \beta_3)=(6, 0.4, -0.1, 0.005)$ in A; $(3.3, 1, 0.2, -0.03)$ in B; $(-5, 1, 0.2, -0.02)$ in C; $(-1.3, 0.5, -0.06, 0.0063)$ in D.

multiple linear regression of y^{-1} on x_1^{-1} and x_2^{-1}; similarly an additive model involving x_1^{-1}, x_2^{-1}, x_1 and x_2 can be found from (3.3) only when β_{00}, β_{20}, β_{02} and β_{22} are all zero.

In order to understand the types of surface which can arise, we may imagine taking a cross-section in the direction of x_1 at each of several fixed values of x_2.

4. Estimation of parameters

Nelder[2] proposed two methods of estimating the parameters $\boldsymbol{\beta}$. The first method, weighted least squares, gives good first approximations for use in the second method, which obtains the maximum

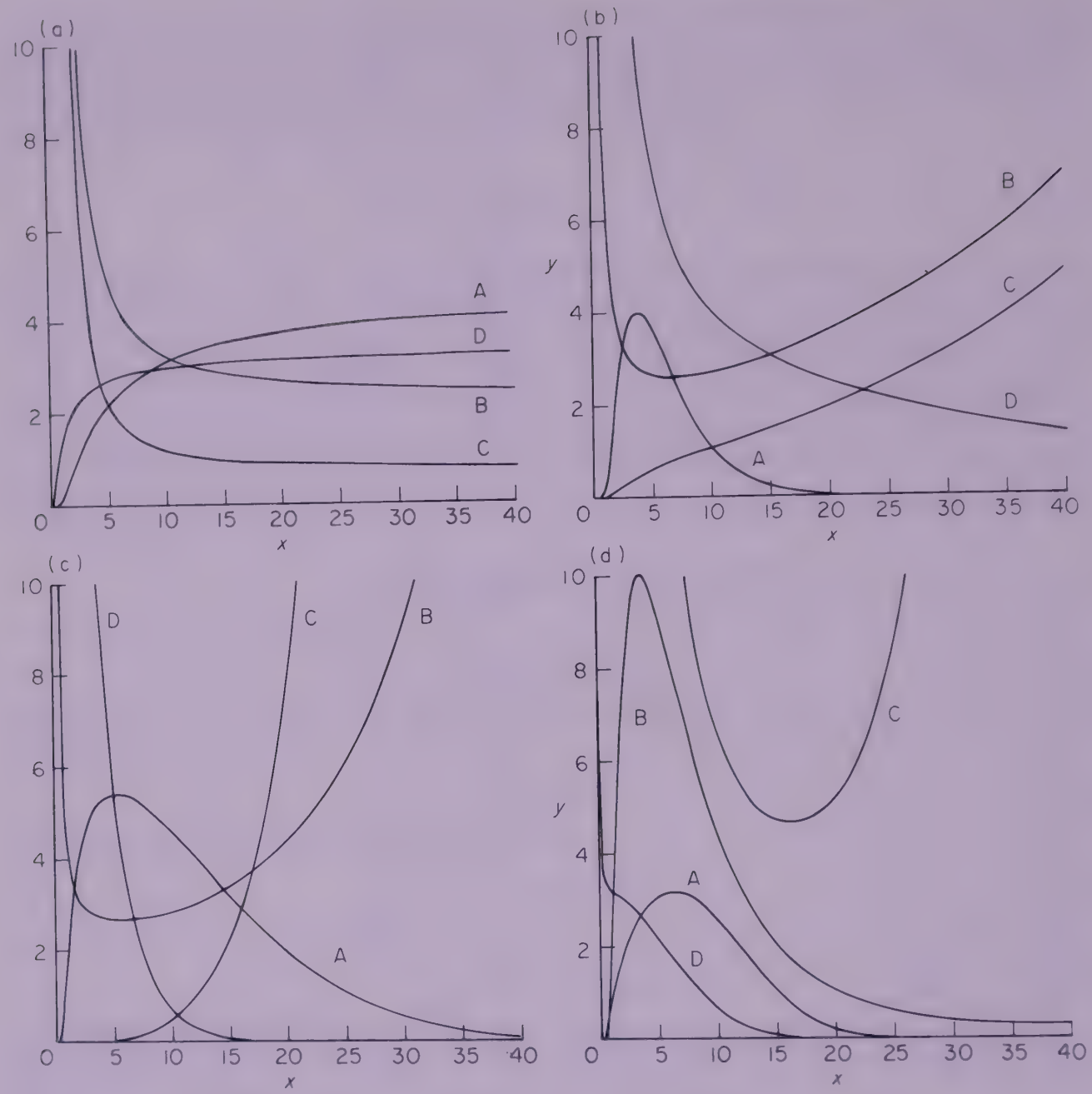

Figure 2. Graphs of the function $y=\exp(\beta_0/x+\beta_1+\beta_2 x+\beta_3 x^2)$. (a) $\beta_2=\beta_3=0$. A: $(\beta_0,\beta_1)=(-3.6, 1.5)$. B: $(\beta_0,\beta_1)=(3.9, 0.8)$. C: $(\beta_0,\beta_1)=(6, -0.4)$. D: $(\beta_0,\beta_1)=(-1, 1.2)$. (b) $\beta_3=0$. $(\beta_0,\beta_1,\beta_2)=(-5, 4, -0.34)$ in A; $(1.5, 0.5, 0.035)$ in B; $(-4, 0.08, 0.04)$ in C; $(4.5, 1.2, -0.025)$ in D. (c) $(\beta_0,\beta_1,\beta_2,\beta_3)=(-1.5, 2.2, -0.033, -0.002)$ in A; $(0.6, 0.8, 0.004, 0.0014)$ in B; $(-23, 0.4, 0.1, 0.002)$ in C; $(0.6, 3.6, -0.4, -0.0004)$ in D. (d) $(\beta_0,\beta_1,\beta_2,\beta_3)=(-1.5, 1.1, 0.13, -0.013)$ in A; $(-3, 4, -0.25, 0.003)$ in B; $(5, 2.8, -0.21, 0.007)$ in C; $(0.06, 1.1, 0.021, -0.0174)$ in D.

likelihood solution using an iterative numerical method. Both methods assume that the variance of an observed response y_l is $\sigma^2(E[y_l])^2$, that is to say its standard error is proportional to its expected value. Under maximum likelihood it is assumed that $\log y_l$ is (asymptotically) normal with parameters $E[\log(y_l)]$, σ^2. Pike[3] observed that, besides the drawbacks mentioned in section 1 above, the ML estimates of $\boldsymbol{\beta}$ are biased. Noting that it is $\log y_l$ about which distributional assumptions are made, we propose in section 5 a model based on $\log y$ which avoids these statistical drawbacks but retains the flexibility of shape which has been the major reason for using inverse polynomials. In practice, the logarithmic transformation is often used to stabilise variances, and the choice between y and $\log y$ in many real sets of data is not at all clear cut. It is shown in section 8 that the proposed model fits data just as well as do inverse polynomials.

5. An alternative model

As in equation 1.3, let $P[x_1, x_2, \ldots, x_k]$ be a polynomial in the levels of the quantitative factors $\mathbf{x}$, and y be the corresponding response. Let $\log y$ be normally distributed with mean $E[\log y]$ and constant variance σ^2 (the same error structure as inverse polynomials).

$$\prod_{i=1}^{k} x_k \log y = P[x_1, x_2, \ldots, x_k] \tag{5.1}$$

will be called a *log-linear response function* (LLRF).

The one-factor first-order LLRF is

$$x \log y = \beta_0 + \beta_1 x \tag{5.2}$$

or, alternatively,

$$\log y = \beta_0/x + \beta_1 \tag{5.3}$$

showing $\log y$ linearly related to $1/x$. Thus, in log-linear models as in inverse polynomials, the first term to be included in the polynomial is always $1/x$. For negative β_0, y approaches 0 as x approaches 0; also as x increases y tends to the asymptotic value $\exp(\beta_1)$. When x is small y is approximately $\exp(\beta_0/x)$. The one-factor second-order LLRF is

$$x \log y = \beta_0 + \beta_1 x + \beta_2 x^2 \tag{5.4}$$

or

$$\log y = \beta_0/x + \beta_1 + \beta_2 x \tag{5.5}$$

and for small x, y is approximately $\exp(\beta_0/x)$; while for large x, y is approximately $\exp(\beta_2 x)$. Hence β_0 and β_2 describe the rising and falling extremes of the curve respectively, provided both β_0 and β_2 are negative. Figures 2a–d show a range of shapes of $y = \exp(\beta_0/x + \beta_1 + \beta_2 x + \beta_3 x^2)$: in (a), $\beta_2 = \beta_3 = 0$ and in (b) $\beta_3 = 0$.

Provided $\beta_0 < 0$, $\beta_2 < 0$ there is a maximum value of y at $x = \sqrt{\beta_0/\beta_2}$; the value is $y = \exp(\beta_1 + 2\sqrt{\beta_0\beta_2})$. Thus β_1 does not affect the position of the maximum but only the value of y taken there. Clearly this family of curves does not have built-in symmetry, the behaviour of the curve at its two extremities being determined by β_0, β_2 respectively.

6. LLRF in two factors x_1 and x_2

The two-factor first-order model is

$$x_1 x_2 \log y = \beta_{00} + \beta_{10} x_1 + \beta_{01} x_2 + \beta_{11} x_1 x_2 \tag{6.1}$$

or, alternatively,

$$\log y = \frac{\beta_{00}}{x_1 x_2} + \frac{\beta_{10}}{x_2} + \frac{\beta_{01}}{x_1} + \beta_{11} \tag{6.2}$$

and the second-order LLRF is

$$x_1 x_2 \log y = \beta_{00} + \beta_{10} x_1 + \beta_{01} x_2 + \beta_{11} x_1 x_2 + \beta_{20} x_1^2 + \beta_{02} x_2^2 + \beta_{21} x_1^2 x_2 + \beta_{12} x_1 x_2^2 + \beta_{22} x_1^2 x_2^2 \tag{6.3}$$

which may also be written

$$\log y = \frac{\beta_{00}}{x_1 x_2} + \frac{\beta_{10}}{x_2} + \frac{\beta_{01}}{x_1} + \beta_{11} + \beta_{20}\frac{x_1}{x_2} + \beta_{02}\frac{x_2}{x_1} + \beta_{21} x_1 + \beta_{12} x_2 + \beta_{22} x_1 x_2 \tag{6.4}$$

In equation 6.2, $\log y$ is bilinearly related to $1/x_1$ and $1/x_2$; y will tend to an asymptotic values when either of x_1 or x_2 is increased and the other is held constant, or also if both are increased in a constant ratio. When $\beta_{00}=0$, we have the simple linear regression of $\log y$ on x_1^{-1} and x_2^{-1}. Like equation 6.4 reduces to an additive regression model only when all of $\beta_{00}, \beta_{20}, \beta_{02}, \beta_{22}$ are zero.

In practice, when y is some measurement of growth or crop yield in plants and $\{x_1, x_2, \ldots, x_k\}$ represent the amounts of fertiliser added while the plants were growing, the simplified version of equation 6.2

$$\log y = \frac{\beta_{01}}{x_1} + \frac{\beta_{10}}{x_2} + \beta_{11}$$

is often inadequate because it contains only the 'main-effect' terms in $1/x_1$ and $1/x_2$. Interactions will very likely occur between the fertilisers, and the full model (6.2) is best understood as the 'linear×linear' set of terms

$$\left(\frac{a_1}{x_1} + b_1\right)\left(\frac{a_2}{x_2} + b_2\right)$$

where a_1, a_2, b_1, b_2 are constants: multiplying this out, and writing $a_1a_2=\beta_{00}$, etc., the full interaction model is obtained.

In the same way the full model (6.4) may be seen as the 'quadratic×quadratic'

$$\left(\frac{a_1}{x_1} + b_1 + c_1x_1\right)\left(\frac{a_2}{x_2} + b_2 + c_2x_2\right)$$

which reduces to a main-effects model only when the coefficients of all the product and ratio terms in x_1, x_2 are zero. 'Linear×quadratic' models can also be used. In fact the number of terms to be used is restricted by the numbers of levels at which factors are included in an experiment: if x_1 has only two levels, then a linear model in x_1 is the most that can be used, while if x_2 has three levels a quadratic is the highest possible for x_2. The full model in this situation is

$$\log y = \frac{\beta_{00}}{x_1x_2} + \frac{\beta_{01}}{x_1} + \frac{\beta_{10}}{x_2} + \beta_{11} + \beta_{12}x_2 + \beta_{02}\frac{x_2}{x_1} \tag{6.5}$$

Adding a 'cubic' term is rarely worth while, even when possible, since it only affects the shape of the right-hand end of the response curve, not the position of the optimum or the rate of approach to the optimum which are likely to be the important measures.

7. Estimation in LLRF

It is convenient to write

$$E[\log y_l] = \sum_{j=0}^{p} \beta_j u_{lj} \tag{7.1}$$

in which $\{y_l: l=1, 2, \ldots, n\}$ are observed responses, $\text{var}[\log y_l]=\sigma^2$ (constant), and u_{lj} is the lth value of $u_j(\mathbf{x})$: the $\{u_j\}$ are the various expressions in $x_1, x_2, \ldots, x_k$ which appear as terms in the model, as for example in equation 6.2, $(u_1, u_2, u_3, u_4)\equiv(x_1^{-1}, x_2^{-1}, x_1^{-1}, 1)$ and in equation 6.4, $(u_1, u_2, \ldots, u_9)\equiv(x_1^{-1}, x_2^{-1}, x_2^{-1}, \ldots, x_1x_2)$. Also $\log y_l$ is assumed normally distributed.

Estimation of $\boldsymbol{\beta}$ is now achieved by carrying out a multiple linear regression of $\log y$ on $\{u_j\}$: the resulting $\hat{\boldsymbol{\beta}}$ are therefore Minimum Variance Linear Unbiased Estimators, a very desirable property. It is simple to estimate the variance-covariance matrix of $\hat{\boldsymbol{\beta}}$ and to test hypotheses concerning members of $\boldsymbol{\beta}$ by the usual linear regression methods, since the distribution of any member of $\boldsymbol{\beta}$ is now known. On theoretical grounds the LLRF model therefore has much to commend it.

It has been assumed so far that the experimental levels of factors $\mathbf{x}$ applied to plants are exactly

those biologically available for growth. Nelder[2] reported that in field work on crop nutrition allowance must be made for base-levels of fertilisers already present in the soil, which altered the 'origin of measurement' of x; fitting 'origins' adds further parameters to all models, since x_i must be replaced by $x_i+\alpha_i$ throughout. The work first reported by Bould,[4] which forms the example in section 8 below, was carried out in chemically-washed sand in pots, and involved only major nutrients. For this class of experiment, 'origins' are not needed. However, when trace elements are included in nutrition experiments, even in washed sand in pots, it is found necessary to fit 'origins', and in field work with any nutrient the remarks of Nelder[2] apply to fruit crops as to any other crop.

8. Analysis of experimental data

Choice of the number of levels to use in an experiment to be analysed by LLRF, or by inverse polynomials, is guided by any previous knowledge of where a 'plateau' in the response surface may begin, or a maximum occur, and by the probable rate of change of response as **x** changes. Levels need not be equally spaced: a logarithmic scale (0.5, 1, 2, 4) has often proved useful in nutrition work. Ali[5] examined data from a series of fruit nutrition experiments, carried out at the Long Ashton Research Station on black currant, raspberry and strawberry crops. Factors **x** represented amounts of major nutrient elements (nitrogen, phosphorus, potassium, magnesium and others) applied to these crops during growth and fruiting; thus the range of values for each x_i was limited physically to what the plants required for reasonably healthy growth. Analysis must therefore be based on a family of models that is flexible enough to fit several different types of data on growth and crop, over a range of values of **x** that covers normal growth of these fruit plants.

To assess whether a model is a good fit to a set of data, the residual mean square on that model may be compared with the residual mean square obtained by fitting the largest model possible for that particular experiment. Any term whose omission would greatly increase the residual mean square must be retained. LLRF analyses usually lead to models that require many fewer than the maximum possible number of terms. When comparing the fit of LLRF and inverse polynomial models to data, Ali[5] computed the residual mean squares after fitting the same terms $\{u_j\}$ in LLRFs as earlier workers had found necessary for good inverse polynomial fits. Table 1 gives a small selection of results, those for shoot growth and total crop yield in three experiments (numbers 3, 4 and 5 in the Long Ashton series) on black currants: these results are typical of many others which show that LLRF models are good in practice, besides having good statistical properties and being straightforward to compute. A full theoretical discussion of general linear models (which include inverse polynomials and log-linear models) is given by McCullagh and Nelder.[6]

Table 1. Residual mean squares after fitting inverse polynomial and log-linear response functions to data from black currant experiments

Experiment no.	Response	RMS for Full Model[a]	No. of terms in Full Model[a]	RMS for Inverse Polynomial[b]	RMS for LLRF[c]	No. of terms in IP and LLRF models
3	Yield	0.0077	20	0.0082	0.0078	8
	Shoot growth	0.0040	20	0.0037	0.0036	9
4	Yield	0.0102	16	0.0087	0.0089	11
	Shoot growth	0.0018	16	0.0019	0.0019	13
5	Yield	0.0134	36	0.0139	0.0146	8
	Shoot growth	0.0022	36	0.0022	0.0022	12

[a]Full Model is that containing all terms (linear, quadratic etc.) that could be fitted given the numbers of levels of factors used in the experiment.

[b]Best fitting Inverse Polynomial found by previous workers.

[c]LLRF using same terms as best fitting Inverse Polynomial.

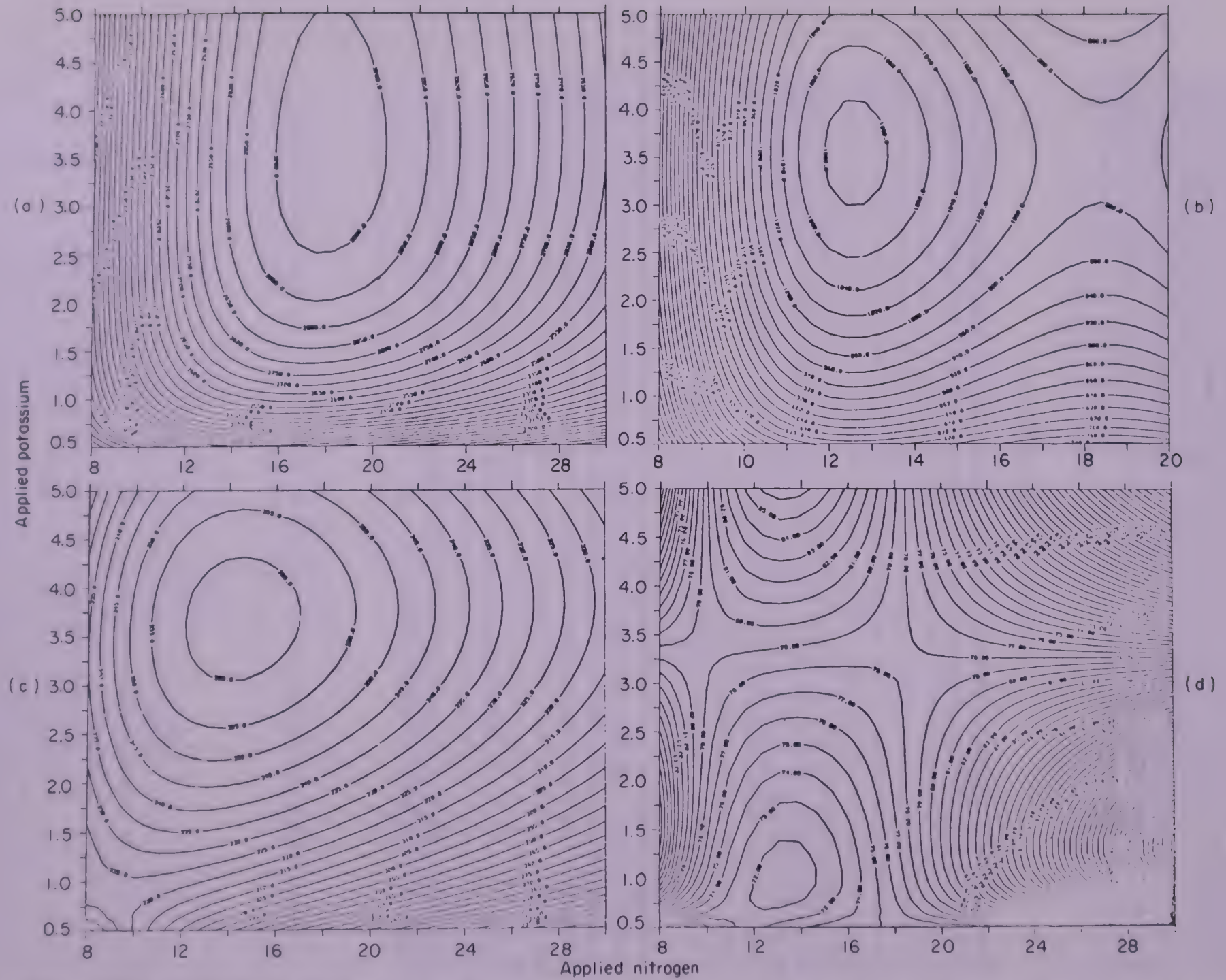

Figure 3. Contour diagrams using the models of Table 2: (a) total crop yield; (b) shoot growth (length in first season); (c) ascorbic acid content of fruit; (d) weight of sample of 100 berries. Horizontal scale: level of applied nitrogen (mEq litre $^{-1}$). Vertical scale: level of applied potassium (mEq litre $^{-1}$).

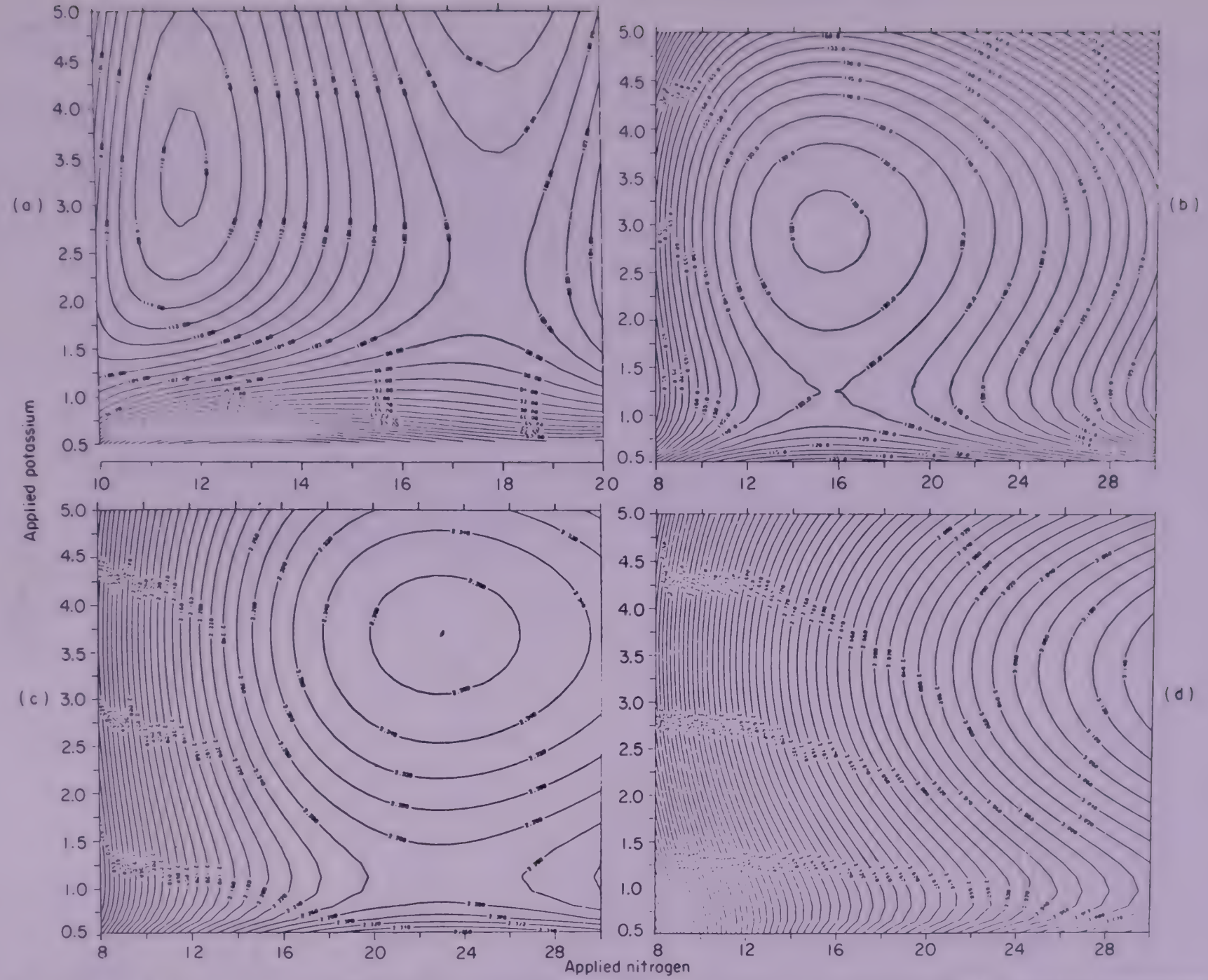

Figure 4. Contour diagrams using the models of Table 2: (a) berry numbers; (b) flower numbers; (c) leaf nitrogen mid-season ($\mu g\ g^{-1}$ of dry matter); (d) leaf nitrogen at end of season ($\mu g\ g^{-1}$ of dry matter).

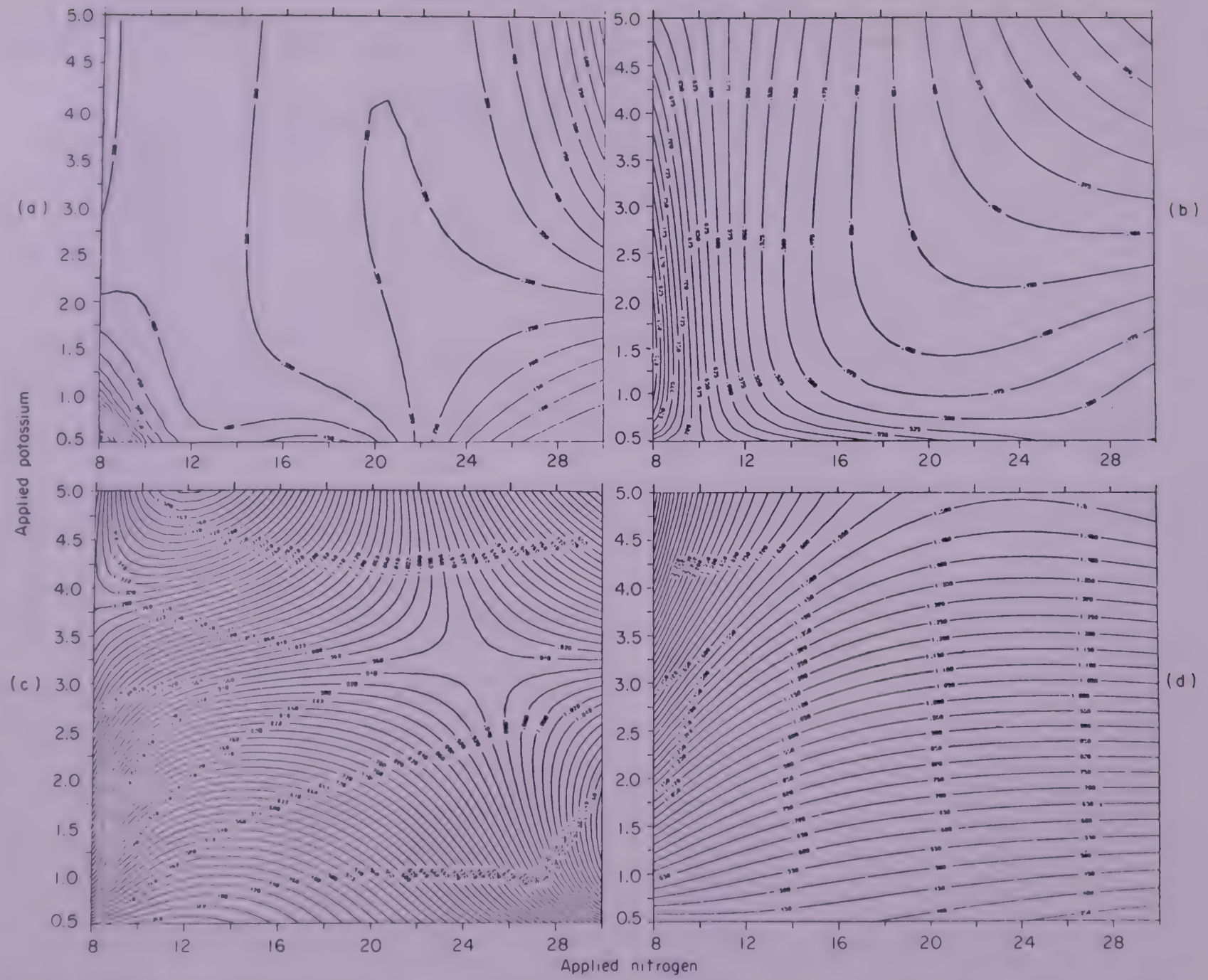

Figure 5. Contour diagrams using the models of Table 2: (a) leaf phosphorus, mid-season; (b) leaf phosphorus, end of season; (c) leaf potassium, mid-season; (d) leaf potassium, end of season; (all in $\mu g\ g^{-1}$ of dry matter.)

9. Detailed study of fitted models

When a good model has been found, contour diagrams of crop yield (or other response) may be plotted against levels of two of the applied factors, as in Figures 3–5. When a response has a maximum the contours indicate this, and show also how rapidly its value falls away from the maximum as factor levels change. There may not be a maximum, in the mathematical sense, within the physically reasonable range of factor levels; or the response may approach a 'plateau' and change very slowly. As some of the following examples show, other considerations (e.g. economic) may then have more influence on recommendations of 'best' factor levels.

Besides crop yield, other measures of general plant health, especially leaf chemistry, may be available. In the series of experiments on black currants carried out at Long Ashton Research Station and reported by Bould,[4] various physiological measurements were made (see below) and ascorbic acid—vitamin C—was also measured in the fruit. When using the response surfaces as a guide to the best levels of fertiliser to apply to the plants, some consistency is hoped for between the results for the various different measures of health. Experiment 3 in the series had nitrogen included at 10, 14, 16, 18 and 20 mEq litre^{-1} and potassium at 0.5, 1, 2, 4 mEq litre^{-1} giving twenty treatment combinations; phosphorus and magnesium were both fixed at 4 mEq litre^{-1} and other nutrients at appropriate standard levels in the watering solution. The experiment lasted two seasons: in the first, new cuttings rooted and grew, their shoot growth being measured at the end of the season. The shoots fruited in the second season, crop yield and ascorbic acid being measured. In addition, in the second season leaf nitrogen, phosphorus and potassium were determined (μg g^{-1} dry weight) once in mid-season and once at the end. Flower numbers and berry numbers were recorded on each plot at appropriate times in the fruiting season, and the weight of a sample of 100 berries was measured from each plot at the end.

The most important records were those of crop yield and ascorbic acid, and the appropriate levels of fertiliser to apply in future would be those which maximised these so far as possible. However, any abnormal values of leaf chemistry would be a warning not to assume that plants would remain healthy every year if treated with levels of nitrogen and potassium that had led to these abnormal values, however good crop yield appeared to be in this one season. Thus the optimum fertiliser levels will be those that maximise crop yield and ascorbic acid in healthy plants. Table 2 shows the terms that were needed in each model in order to achieve good fits to the data; N and K are used, rather than x_1, x_2 in the table for greater clarity to denote the levels of applied fertilisers.

The final five columns in Table 2 represent interactions in various forms between N and K. All but three of the recorded items required at least one interaction term to achieve a satisfactory fit, although the important records on crop yield and ascorbic acid do not need a very complicated model. Using the models of Table 2, contour diagrams were drawn on a computer graph plotter; these are shown in Figures 3–5. Units of fertiliser are mEq litre^{-1} throughout.

Table 2. Terms required in log-linear models for data of black currant experiment no. 3

Record	1/N	N	N²	1/K	K	K²	1/NK	K/N	N/K	NK	N²/K
Total crop yield	√	√	—	√	√	—	√	—	—	—	—
Shoot length	√	√	√	√	√	√	—	—	—	—	—
Ascorbic acid	√	√	—	√	√	\	\	—	—	—	—
Weight of 100 berries	√	√	—	√	√	—	\	\	\	\	—
Berry numbers	√	√	\	√	√	—	\	—	\	—	\
Flower numbers	√	√	—	√	√	\	—	—	—	—	—
First sampling date — Leaf N	√	√	—	√	√	\	—	—	—	—	—
First sampling date — Leaf P	√	√	√	√	√	—	√	—	√	—	\
First sampling date — Leaf K	√	√	—	√	√	√	√	√	√	√	—
Second sampling date — Leaf N	√	—	—	√	√	√	√	√	√	\	—
Second sampling date — Leaf P	√	√	—	√	√	—	√	√	√	\	—
Second sampling date — Leaf K	√	√	—	√	√	√	√	—	√	—	—

All models also contain a constant term.

Figure 3a has a maximum a little over 3000 g plot^{-1}, and yield falls away relatively slowly from this, the contours being 50 g plot^{-1} apart. Nitrogen between 16 and 20 and potassium between 2.5 and 5 thus give satisfactory results. Figure 3b indicates that shoot growth is greatest (contour 1080 cm plot^{-1}) for nitrogen between 12 and 13 and potassium between 3 and 4; the potassium level is more critical than the nitrogen (contours are 20 cm plot^{-1} apart). Ascorbic acid (Figure 3c) also has a similar maximum, with nitrogen in the region 12 to 17 and potassium 3 to 4.25: the maximum contour is 360 units and the contours are 5 units apart. These three contour diagrams are reasonably easy to interpret, and they also show that there is some flexibility in the best levels of fertiliser to apply, as the responses do not fall away from their maxima very rapidly. However, Figure 3d is not straightforward: taking a vertical line through N=14, the weight increases steadily from about K=0.75 upwards, but at N=20 the weight is fairly constant for K between 0.5 and 3, and becomes constant again (at a lower level) for K=4 and upwards. In the region of N=16 to 20 (which was best for crop yield) and K=3 to 4 (best for shoot growth and good for crop yield) the weight is not very sensitive to changes in nitrogen and potassium: the contour lines are 1 g apart. The model for berry weight, it will be noted, contained more interaction terms than the others.

In Figure 4a, there is a region of maximum response (118) for nitrogen about 12 and potassium between 2.75 and 4; contours are 2 units apart and so we can see that the response falls slowly as nitrogen is increased, being influenced little by the level of potassium provided this is above 2. Figure 4b has a minimum (*not* a maximum) between N=14 and 17, K=2.5 to 3.25; this may seem to conflict with Figure 4a, but clearly represents differential fruit setting. As contours are 5 units apart this response is changing relatively quickly.

The leaf chemistry remains for study. At the mid-season sampling date, leaf nitrogen (Figure 4c) shows a maximum value of 2.38 μg g^{-1} at about N=23, K=3.75: the 2.36 contour extends from N=20 to 25 and K=3.25 to 4.25, and the further away we move from the maximum the closer the contours become (they are at intervals of 0.02 μg g^{-1}). By the end of the season (Figure 4d), leaf nitrogen is steadily increasing even beyond N=30, which is not a level that is very likely to be used in an experiment; for the range 12 to 20 it is affected little by the level of applied potassium—though a little higher between K=3 and 4 than elsewhere. Excess nitrogen continues to be deposited in the leaf at this stage.

Figure 5a indicates that, over the experimental ranges of nitrogen and potassium, leaf phosphorus in mid-season remains in the range 0.3 to 0.35 with little variation, while from Figure 5b it is seen that the contours (at intervals of 0.025) rise from top right to bottom left in the diagram. The levels when applied nitrogen and potassium are low are considerably above what is expected in healthy plants, and by this stage in the season excess phosphorus may be being deposited in small leaves.

As regards leaf potassium, Figure 5c has contours at intervals of 0.02, and there is a 'saddle' at approximately N=25, K=3.25. The mid-season value increases from this saddle as K rises above 3.25 and N decreases below 25. In healthy plants, a value above 1.000 is desirable so the region giving optimum crop yield also gives suitable leaf potassium values. By the end of the season the contour pattern (Figure 5d) has changed considerably, the amount of leaf potassium now depending almost entirely on the amount of potassium applied to the plants, leaf values above 1.000 requiring applied potassium above about 2.75.

9.1. Discussion of experiment 3

Since yield and ascorbic acid are the most important records, we appear to be able to satisfy the aim of the experiment by recommending nitrogen to be applied at 16–17 mEq litre^{-1} (the lower end of the best range for yield and the upper end of that for ascorbic acid). Berry weight may also be important when fruit is sold fresh rather than for processing, and 16 mEq litre^{-1} is an acceptable value for applied nitrogen to satisfy this. Lower N levels increase berry numbers, but this is not particularly desired; and shoot growth and flower numbers are only of secondary importance for their bearing on yield. A firm recommendation of N=16 is therefore possible, and it does not lead to leaf chemistry values that conflict with ideas of general plant health.

For applied potassium, the range of values leading to satisfactory yield, ascorbic acid and berry

weight is approximately 3.5 to 4.25, and suitable leaf chemistry values result from the lower part of this, say 3.5 to 4. If an easy and convenient value is required for future use, K=4 can be recommended for application, though economically there may be a case for reducing this to K=3.5 as only berry weight would be much less than optimum under this change.

The applied levels of phosphorus and magnesium were both fixed at 4 mEq litre $^{-1}$ in this experiment; analysis of other experiments in the series shows these are quite near to optimum values. The use of log-linear models has thus given a very informative analysis that can be carried out using an ordinary regression program on a computer, taking log y as dependent variable and including terms in $1/x$ in the model.

Acknowledgement

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Body Composition and Feed Intake of Young Pigs Postweaning

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Thirty-five female Large White pigs were weaned at 14 days of age, penned individually, given a single diet and slaughtered serially at 25, 40, 55 and 70 days of age. A control group was fed to appetite throughout while three groups were subjected to feed restriction at various ages postweaning. For the periods 25 to 40, 40 to 55, 55 to 70 days of age pigs fed to appetite gained 321 (±19.9), 532 (±35.4) and 601 (±35.5) g liveweight day^{-1}. Pigs restricted between 25 and 55 days, followed by refeeding from 55 to 70 days, gained 192 (±14.0), 169 (±44.8) and 508 (±81.0) g liveweight day^{-1}; pigs restricted between 40 and 55 days, followed by refeeding from 55 to 70 days, gained 425 (±42.8), 409 (±49.6) and 480 (±70.1) g liveweight day^{-1}. A group given 200 g feed day^{-1} gained 42 (±4.2) g liveweight daily between 25 and 70 days. Refed groups did not differ significantly from controls in their feed intakes for the period 55 to 70 days, either in absolute intakes or in feed intake kg^{-1} mean liveweight. Refed pigs grew no faster, and made no greater protein gains than controls of equivalent weight; however, lipid gains were elevated.

Keywords: Compensatory growth; body composition; feed intake; young pigs.

1. Introduction

Removal of pigs from the sow at weaning causes a reduction in feed intake resulting in weight stasis or weight loss.[1] The rate and composition of liveweight gain when feed intake subsequently increases will be influenced by the age at which the weight check takes place, its severity and duration. In such circumstances the ability of the young animal to compensate by exhibiting catch-up growth,[2] particularly catch-up protein growth, is of both biological and commercial significance. However, recent studies[3,4] suggest that the phenomenon may not exist in young, rapidly-growing animals.

For catch-up growth to occur following a voluntary or imposed intake restriction one or more of three circumstances ought to prevail. First, previously-restricted animals could show enhanced feed intakes (in absolute terms or on a kg^{-1} liveweight basis) and thereby accrue more rapid empty body weight gains than unrestricted controls. Or, second, previously-restricted animals could consume the same quantity of food as controls but also realise more rapid empty body weight gains as the result of temporarily lowered maintenance requirement, thus freeing more net energy for growth.[5] Or, third, previously-restricted animals could consume the same quantity of food as controls but achieve more rapid empty body gains because the tissues deposited were of lower energy density, containing relatively more protein and water and less lipid.[6]

This experiment investigated short-term growth responses by young pigs to refeeding following age-based intake restrictions. Compensatory growth experiments are not easy to conduct, no doubt adding to the controversy surrounding the phenomenon. This experiment used a design which obviated some of the problems posed by weight-based experiments, but generated difficulties of its own, some of which followed from the restricted scale of the trial. Nevertheless, at a time when some extension specialists may propose commercial benefit from a period of feed restriction early in the pig's productive life, this study suggests the value of the contrary strategy.

2. Experimental

Large White females were weaned at 14 days of age and 2.7–4.8 (s.d. 0.72) kg liveweight into tiered cages. After seven days on *ad libitum* intake of an early-weaning diet (Table 1), 35 pigs, now weighing 3.5–6.9 (s.d. 0.83) kg liveweight, were transferred to individual cages fitted with nipple drinkers and troughs designed to minimise spillage. Room temperature was gradually reduced from 27°C (at 14 days of age) to 24°C (at 70 days of age). Between 21 and 25 days of age all pigs were fed to appetite on the early-weaning diet; 'to appetite' feeding involved the provision of three discrete 'meals' per day, the quantity allowed being increased slightly when the previous 'meal' had been consumed entirely. Previous experience at this centre has indicated that this method of feeding young pigs promoted greater feed intakes than those obtained using an *ad libitum* feed hopper. The experimental design is shown in Table 2. The early-weaning diet (Table 1) was used throughout.

After slaughter the gut contents were removed and the whole empty body minced, twice through a 13 mm plate and once through a 5 mm plate. Gross energy (GE) was determined by adiabatic bomb, nitrogen by Kjeldahl digestion and lipid by use of the equation:

$$\text{Lipid}=(\text{GE}-0.1475\text{N})/0.0393.^{1}$$

Table 1. Composition of early-weaning diet

Ingredient	(g kg^{-1} fresh weight)
Ground barley	100
Ground wheat	100
Ground maize	250
Soya bean meal	100
Fishmeal (Provimi 66)	150
Whey powder	200
Mineral and vitamin mix	50
Tallow[a]	50
DM (g kg^{-1} fresh weight)	910
Chemical analysis (kg^{-1} DM)	
Nitrogen (g)	36.7
Ether extract (g)	87.4
Fibre (g)	28.0
Ash (g)	93.4
Gross energy (MJ)	19.4
Digestible energy (MJ)[b]	15.8
Digestible nitrogen (g)[b]	33.3

[a] 'Priplus 45' fully hydrogenated tallow flake, Unichema International, Bebington, UK.

[b] Determined by live pig balance technique.

Table 2. Experimental design, together with feed intake

	Group			
	I	II	III	IV
Number of pigs, total	14	9	6	6
Slaughtered, 25 days of age	4	—	—	—
Intake, 25 to 40 days	A (0.945)	R1 (0.736)	A (0.923)	R2 (0.459)
Slaughtered, 40 days of age	4	3	—	—
Intake, 40 to 55 days	A (0.847)	R1 (0.536)	R3 (0.805)	R2 (0.394)
Slaughtered, 55 days of age	3	3	3	3
Intake, 55 to 70 days	A (0.872)	A (0.858)	A (0.821)	R2 (0.347)
Slaughtered, 70 days of age	3	3	3	3

A, Fed to appetite, three times daily; R1, daily feed intake restricted to feed intake at 25 days of age; R2, daily feed intake restricted to 200 g diet; R3, daily feed intake restricted to feed intake at 40 days of age.

Numbers in parentheses refer to the achieved 15-day feed intakes per kg mean liveweight.

Within treatment groups, carcass compositions of pigs at each slaughter point were used in regression analysis to estimate the relative proportions in the liveweight at slaughter of empty body, water, nitrogen, lipid and ash; these proportions were then used to estimate the carcass composition of pigs in the same treatment group which were not slaughtered. Daily gains in liveweight and body components, daily feed intakes, feed intakes relative to mean liveweight and carcass compositions were examined by analysis of variance.

3. Results

Changes in liveweight during the experiment are shown in Figure 1. Mean liveweights at 40 days of age were 9.8, 8.0, 12.2 and 6.8 kg (s.e.d. 1.05) for groups I, II, III and IV respectively; at 55 days group means were 18.0, 10.5, 18.4 and 7.7 (s.e.d. 1.80) and at 70 days, 27.8, 19.5, 28.7 and 7.7 kg (s.e.d. 3.65). Carcass compositions of pigs slaughtered at 25, 40, 55 and 70 days are given in Table 3. The initial slaughter group of four pigs at 25 days comprised 0.74 water, 0.065 lipid, 0.15 protein and 0.035 ash in the empty body. Differences between carcass compositions of pigs from groups I and II at 40 days failed to reach significance, despite the fact that for the previous 15 days group I pigs had been fed to appetite while group II pigs had been restricted to their 25-day intake. By 55 days of age severely-restricted pigs in groups II and IV had significantly less mass of empty body and body components than their appetite-fed contemporaries in group I ($P<0.01$). Mildly-restricted pigs in group III (held to their 40-day intake for the preceding 15 days) were found to have carcass compositions intermediate to those of groups I and II. Lipid content of empty body was a particularly variable character, irrespective of dietary treatment.

Group weights for empty body protein and lipid are depicted in Figure 2.

Following 15 days of refeeding, 70-day carcass compositions for group III pigs resembled those of appetite-fed controls. A more severe restriction prior to refeeding (group II) produced significantly smaller empty body and chemical component masses at 70 days than those of groups I and III. By this final slaughter age, pigs fed 200 g per day for the preceding 45 days weighed 0.28 of control mean liveweight and contained only 0.024 lipid in the empty body.

Daily gains of body components are given in Table 4. In the course of the latter 15 days of their 30-day intake restriction, group II pigs were only narrowly in positive lipid balance (0.99 g lipid

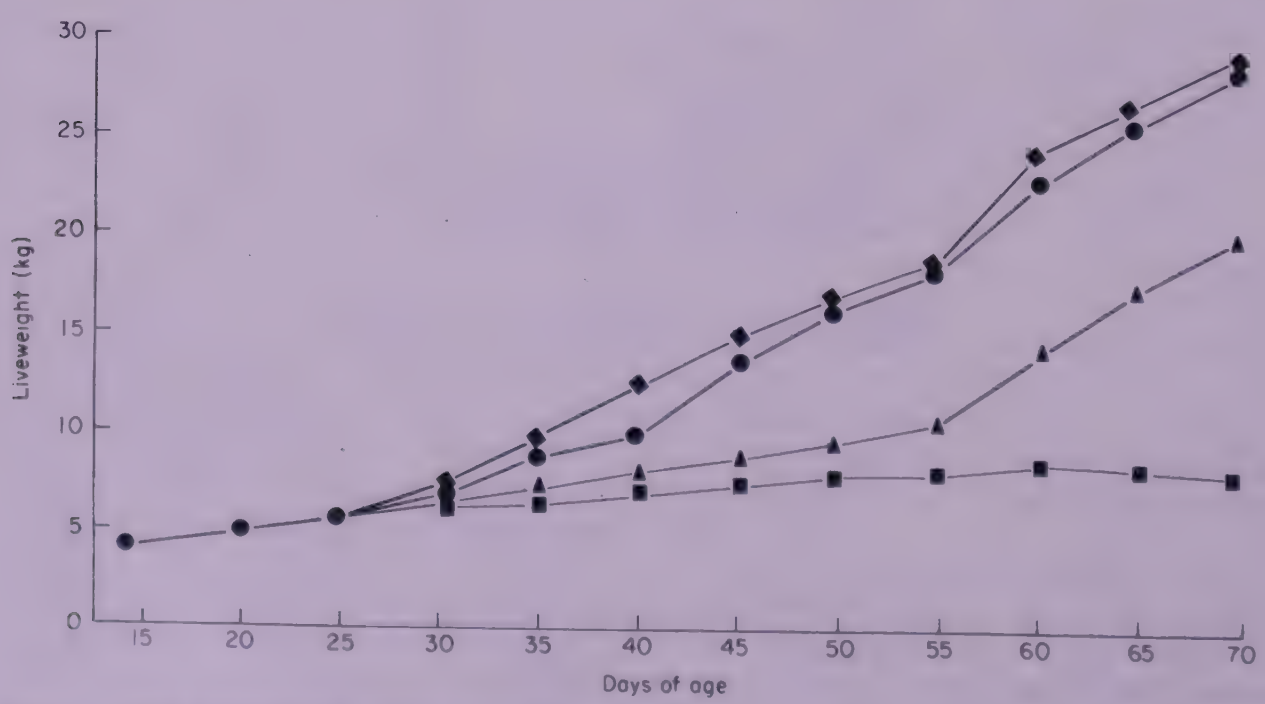

Figure 1. Change in liveweight (kg) between 14 and 70 days by female pigs fed to appetite (group I ●—●), severely restricted and refed (group II, ▲ ▲), mildly restricted and refed (group III, ◆—◆) and severely restricted (group IV, ■—■). Thin lines denote periods of feed restriction, thick lines represent feeding to appetite.

Table 3. Empty body composition at 25, 40, 55 and 70 days of age of appetite-fed, restricted, and restricted and refed female pigs (all weights in grammes)

Days of age	Group I	II	III	IV	Standard error of mean difference	Significance of difference
25						
n	4					
Liveweight	4978					
Empty body weight	4410					
Water	3259					
Lipid	286.5					
Protein	660.0					
Ash	153.3					
40						
n	4	3				
Liveweight	9550	8044			2334.7	n.s.
Empty body weight	8525	6827			2058.9	n.s.
Water	5624	4888			1329.5	n.s.
Lipid	757.2	434.5			403.09	n.s.
Protein	1685.6	1225.0			330.4	n.s.
Ash	346.9	252.0			67.64	n.s.
55						
n	3	3	3	3		
Liveweight	17095	8963	15183	7216	2495.9	**
Empty body weight	15318	8422	14267	6967	2201.1	**
Water	10159	6172	9521	5104	1421.3	**
Lipid	1516.0	428.1	1293.4	260.5	430.92	*
Protein	2961.9	1419.4	2777.5	1295.0	353.25	**
Ash	563.7	317.3	534.1	305.2	72.30	**
70						
n	3	3	3	3		
Liveweight	27750	19467	28733	7718	2495.9	**
Empty body weight	25333	17233	26500	7213	2201.1	**
Water	16922	11649	17359	5240	1421.3	**
Lipid	2992.9	1846.5	2814.3	171.1	430.92	*
Protein	4269.4	3035.0	4653.1	1364.4	353.25	**
Ash	850.9	545.0	996.3	316.6	72.30	**

n.s.=not significant, $*P<0.05$, $**P<0.01$, $***P<0.001$.

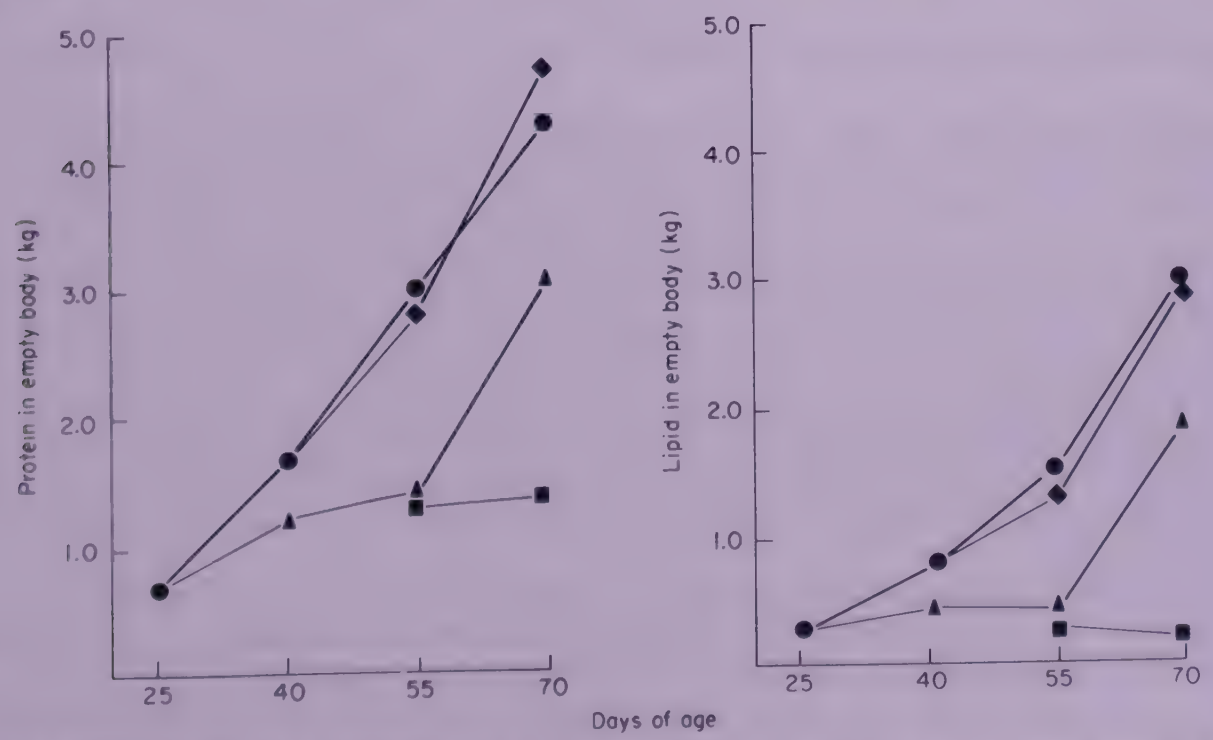

Figure 2. Protein and lipid (kg) in the empty body at 25, 40, 55 and 70 days of female pigs fed to appetite (group I, ●—●), severely restricted and refed (group II, ▲—▲), mildly restricted and refed (group III, ◆—◆) and severely restricted (group IV, ■—■). Thin lines denote periods of feed restriction; thick lines represent feeding to appetite.

Table 4. Daily gains of liveweight, empty body weight, water, lipid, protein and ash between 25 and 40, 40 and 55, 55 and 70 and 25 and 70 days of age by appetite-fed, restricted and restricted and refed female pigs (all weights in grammes)

Days of age	Group I	II	III	IV	Standard error of mean difference	Significance of difference
25–40						
n	4	3				
Liveweight	312	192			38.9	***
Empty body weight	274.4	154.5			32.70	**
Water	204.6	104.1			19.31	**
Lipid	30.7	9.3			7.19	*
Protein	63.3	36.4			10.19	*
Ash	12.4	6.4			1.89	**
40–55						
n	3	3	3			
Liveweight	532	169	409		65.8	***
Empty body weight	440.1	129.2	331.7		44.0	**
Water	296.1	102.3	228.4		24.59	**
Lipid	48.1	1.0	29.7		16.11	***
Protein	81.8	16.7	61.8		10.78	**
Ash	13.1	5.1	10.2		1.70	**
55–70						
n	3	3	3	3		
Liveweight	601	508	480	−23	116.1	**
Empty body weight	563.7	396.2	419.5	−25.8	87.50	***
Water	381.9	225.9	262.3	−21.8	61.70	**
Lipid	87.4	83.9	67.4	−7.5	33.70	
Protein	67.4	75.4	48.2	−3.2	12.62	**
Ash	14.8	8.2	15.9	−1.0	4.53	*
25–70						
n	3	3	3	3		
Liveweight	507	317	490	42	68.0	***
Empty body weight	463.7	277.1	457.9	45.4	58.00	***
Water	302.8	180.8	298.8	31.7	38.30	***
Lipid	60.0	34.1	56.2	−3.7	13.75	**
Protein	79.9	51.5	83.6	13.0	7.66	***
Ash	15.0	8.4	17.6	3.0	1.79	***

n.s.=not significant, $*P<0.05$, $**P<0.01$. $***P<0.001$.

gained day^{-1}, 40–55 days of age). The milder restriction imposed on group III pigs betwen 40 and 55 days of age served to reduce daily gains of empty body, water and lipid ($P<0.01$), but did not significantly curtail protein and ash gains. Subsequent refeeding between 55 and 70 days of age produced gains of remarkably similar mass and chemical composition in groups I and II.

Considering daily gains for the experimental period as a whole (25 to 70 days of age), gains in liveweight and body components were unaffected by a 15-day restriction imposed at 40 days of age (group III compared to group I). However, a 30-day restriction from 25 days of age (group II) resulted in significantly reduced daily gains of liveweight and body components ($P<0.001$) by comparison with gains made by both group I controls and mildly-restricted and refed group III pigs. In response to their severe and prolonged intake restriction group IV pigs suffered a continuous reduction in lipid reserves concurrent with small daily gains in liveweight, empty body weight, water, protein and ash.

Feed intakes per kg mean liveweight between 25 and 40, 40 and 55, 55 and 70, and 25 and 70 days are shown in Figure 3. Refed pigs ate the same per kg mean liveweight as control contemporaries of greater liveweight, and also ate the same per kg mean liveweight as control pigs at equal liveweight but a younger age; feed intake per kg liveweight for control pigs was similar in all three periods of the experiment.

Efficiencies of protein utilisation [protein deposited (g day^{-1})/protein consumed (g DCP day^{-1})] for the period 55 to 70 days were 0.24, 0.42 and 0.17 (s.e.d. 0.080) for groups I, II and III

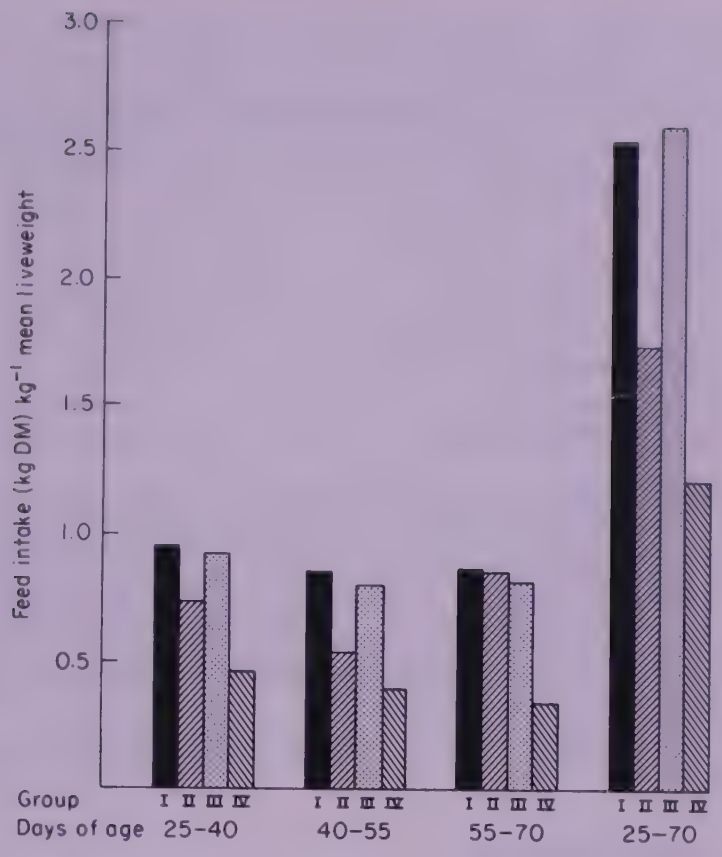
3.0
2.5
2.0
1.5
1.0
0.5
Feed intake (kg DM) kg⁻¹ mean liveweight
Group I II III IV I II III IV I II III IV I II III IV
Days of age 25–40 40–55 55–70 25–70

Figure 3. Feed intake (kg DM) kg^{-1} mean liveweight between 25 and 40, 40 and 55, 55 and 70 and 25 and 70 days of age by female pigs fed to appetite (group I, ■), severely restricted and refed (group II, ▨), mildly restricted and refed (group III, □) and severely restricted (group IVm ▧).

respectively. Efficiencies of energy utilisation [energy deposited (MJ day^{-1})/energy consumed (MJ DE day^{-1})] during the same period were 0.23, 0.35 and 0.20 (s.e.d. 0.057). Considering the total time on test (25 to 70 days of age) dietary protein was utilised with efficiencies of 0.41, 0.53 and 0.40 (s.e.d. 0.049) by unrestricted controls, severely-restricted and refed pigs and mildly-restricted and refed pigs respectively, while dietary energy was converted to energy in the gain with efficiencies of 0.29, 0.34 and 0.27 (s.e.d. 0.040).

4. Discussion

Liveweight gain *per se* was not found to be more rapid in previously-restricted pigs than in controls of the same age or similar empty body weight.

There was a 6.9 kg discrepancy in empty body weight at 55 days of age between pigs in groups I and II, which increased to an 8.1 kg difference at 70 days. Refed young pigs in a comparative experiment by Lodge *et al.*[3] failed to diminish control pigs' advantage in live weight, and no acceleration in rate of whole body protein deposition was detected during realimentation.

Differences in carcass composition between appetite-fed (group I) and severely-restricted (groups II and IV) pigs were not significant until 55 days of age, when restricted pigs had undergone 30 days of intake limitation. At 55 days, slaughtered pigs from groups I and IV contained 0.099 and 0.037 lipid in the empty body respectively; corresponding group means for empty body protein were 0.193 and 0.186. The relative stability of empty body protein under diverse feeding regimes is not unique to the present experiment.[7,8] Recovery of lipid reserves, depleted after weaning at 14 days of age, was protracted. Lipid content of suckled pigs is approximately 0.15 of the empty body;[1] this was reduced to 0.065 empty body by 25 days of age and rose gradually in group I pigs to 0.118 at 70 days. Therefore, even after 55 days of appetite feeding, weaned pigs had not regained their preweaning lipid reserves. Concurrent with this moderate rate of lipid deposition were daily protein gains (80 day^{-1}, group I pigs, 25 to 70 days of age) which compared favourably with values in the literature for pigs of similar age and live weight.[9,10]

When given the opportunity to express appetite after a severe and prolonged intake restriction, group II pigs gradually increased their daily feed intake over a period of 7 days. Mean daily feed intake from 55 to 70 days by refed group II pigs was 903 (±185.1) g DM at a mean empty body weight

of 12.83 kg, which can be assessed in the light of daily intakes of 753 (±38.7) g DM day^{-1} by control pigs of similar empty body weight (group I, 40 to 55 days, mean empty body weight 11.92 kg). On a live weight basis (Figure 3 and Table 2), pigs previously restricted never exceeded intakes achieved by control pigs of the same age or empty body weight fed *ad libitum*. This result corroborates that of Vanschoubroek, *et al.*[11] and for chickens by Wilson and Osbourn,[12] but is contrary to enhanced feed intakes measured in realimented young female pigs by Ratcliffe and Fowler[13] and in older refed pigs offered a high-density diet.[14]

Daily lipid deposition rate for group II pigs during refeeding resembled that of group I pigs of the same age (83.9 *vs* 87.4 g day^{-1}, 55 to 70 days) but considerably exceeded lipid gains by control pigs of similar empty body weight (83.9 g day^{-1}, group II, 55 to 70 days *vs* 48.1 g day^{-1}, group I, 40 to 55 days). Protein gains were comparable at 75.4 and 81.8 g day^{-1}. It appears then that the composition of empty body weight gain during refeeding comprised more lipid than that of control pigs of similar mean empty body weight but a younger age.

There was no significant advantage in efficiency of dietary protein and energy utilisation by young pigs from feeding regimes which incorporated periods of intake restriction over a regime which sought to maximise feed intake following weaning. The slight (non-significant) enhancement of dietary protein and energy utilisation shown by the severely restricted group may be worth further academic study; but as this was achieved at the cost of a substantial liveweight reduction at 70 days, it is of no practical significance.

Dependence upon compensatory growth phenomena in pig feeding strategies postweaning is therefore unwise.

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The Saponin Content and Sapogenol Composition of the Seed of 13 Varieties of Legume

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The total saponin content of various legume seeds together with their sapogenol composition have been determined. Extraction of the defatted flours was effected with methanol and subsequent acid hydrolysis yielded the soyasapogenols which were analysed using gas and thin-layer chromatography. The saponin levels ranged from 0–0.65% for defatted seed while the major sapogenol present in these saponins was soyasapogenol B. The figures obtained are compared with those previously reported and reasons are suggested for the discrepancies.

Keywords: Saponin; soyasapogenol; legume; analysis; t.l.c.; h.p.t.l.c.; g.c.

1. Introduction

Saponins are secondary plant metabolites containing a carbohydrate moiety (mono- or oligosaccharide) attached to an aglycone, which may be steroidal or triterpenoid in structure.[1] Although saponins are widely distributed in the plant kingdom and have been found in animals such as starfish they have been reported in only a relatively small number of food plants, primarily legumes.[2] The significance of the occurrence of saponins in foods rests mainly on their biological activities, either beneficial (e.g. cholesterol lowering[3]) or deleterious.[1,4] Oakenfull[2] has reported that saponins occur to a significant extent in only 17 varieties of plants which are used in any quantities as human food ingredients. Saponin levels were reported for some of these materials, but in the light of recent advances in the quantitative analysis of saponins,[5–7] these figures have been questioned in the case of soya and, by implication, in other legumes.[6,8]

Techniques which have recently been used for the quantitative analysis of saponins include high-performance liquid chromatography[5] and thin-layer chromatography[6] of the intact glycosides and gas–liquid chromatography[6,9] or high-performance liquid chromatography[7] of the corresponding aglycones (sapogenols), released as a result of acid hydrolysis. As none of these techniques has been applied to the analysis of saponins in food plants generally the seed of 12 varieties of legume, together with peanut and etiolated bean sprout, have been examined for saponins using thin-layer and gas–liquid chromatography. These methods are complementary, yielding qualitative and quantitative information about the saponin composition.

2. Methods

2.1. Materials

The legume seed samples examined comprised: field bean *(Vicia faba)*, scarlet runner bean *(Phaseolus coccineus)*, kidney bean *(Phaseolus vulgaris)*, mung bean *(Phaseolus aureus)*, butter bean *(Phaseolus lunatus)*, soya bean *(Glycine max)*, lentil *(Lens culinaris)*, haricot bean *(Phaseolus vulgaris)*, green pea *(Pisum sativum)*, yellow split pea *(Pisum sativum)*, chick pea *(Cicer arietinum)* and jack bean *(Canavalia ensiformis)*. In addition samples of peanut *(Arachis hypogaea)* and

etiolated commercial bean sprouts were examined. All of the samples were obtained locally. All solvents used for extraction were of analytical grade and redistilled before use.

2.2. Extraction

The individual legume seeds were ground to a flour in a mechanical pestle and mortar and 30 g of each was extracted with chloroform (800 ml) for 16 h in a Soxhlet extractor. The chloroform extract was evaporated *in vacuo* to give a measure of the lipid content of the flours. The air-dried defatted flours were then extracted with methanol (800 ml) for 30 h. The methanol extract was evaporated to dryness *in vacuo*, dissolved in distilled water (15 ml) and applied to a column of reversed phase octasilane (C_8) bonded to silica gel (20 g, J. T. Baker Chem. Co., Phillipsburg, NJ). The column was successively eluted with distilled water (150 ml) and methanol (150 ml). Gas chromatographic analysis revealed the water fraction to contain no detectable saponins and this was discarded. The methanol fraction was evaporated to dryness.

2.3. Thin-layer chromatography of intact saponins

The evaporated saponin-containing methanol extract (section 2.2) was taken up in methanol (at a concentration equivalent to 1 g defatted flour ml^{-1}). Aliquots (5 μl) were spotted on to plates (20×20 cm) and developed in the following solvent systems. (a) Silica gel; l-butanol:ethanol:ammonia (0.880), 7:2:5. (b) Silica gel pretreated with oxalic acid; chloroform:methanol:water, 65:35:10 (lower layer).[9] (c) Reversed phase (C_{18}) bonded to silica; methanol:water, 3:2.

All plates were developed for a distance of 12 cm, air dried and sprayed with *p*-anisaldehyde:glacial acetic acid:concentrated sulphuric acid reagent (1:100:2). Individual spots were visualised with gentle heating with hot air, purple/blue colours being characteristic of steroids and triterpenes.

2.4. Acid hydrolysis of saponin fractions

Half of the saponin-containing methanol fraction (section 2.3) was evaporated to dryness in a pear-shaped flask (25 ml) and dried over phosphorus pentoxide in a vacuum desiccator overnight. A solution of dry hydrochloric acid in methanol (5 ml, 5% solution) was then added and the solution refluxed for 3 h. After cooling, the mixture was neutralised with aqueous ammonium hydroxide (2 M) and evaporated to dryness. After redissolving in water (5 ml), the sapogenols were extracted with ethyl acetate (3×5 ml). The combined ethyl acetate extracts were dried over anhydrous sodium sulphate, filtered and evaporated to dryness.

2.5. Thin-layer chromatography of sapogenols and saponin hydrolysis products

The dried sapogenol hydrolysis products (section 2.4) were dissolved in methanol (concentration equivalent to 1 g defatted flour ml^{-1}). Aliquots (2 μl) were spotted on to high-performance thin-layer chromatography plates (10×10 cm, silica, BDH-Merck, Poole, Dorset) together with standards of soyasapogenols A and B and developed in the h.p.t.l.c. tank (Camag, 4132 Muttenz, Switzerland) with chloroform:methanol (96:4) for a distance of 5.5 cm. The air-dried plates were visualised with the *p*-anisaldehyde reagent as described above.

2.6. Gas chromatography of sapogenols

One millilitre of the methanol solution of hydrolysed saponins (section 2.5), equivalent to 1 g of defatted flour, was evaporated to dryness by a stream of nitrogen in a vial. After further vacuum drying over phosphorus pentoxide (12 h), pyridine (200 μl, dried over molecular sieve) and bis(trimethylsilyl)trifluoroacetamide (BSTFA, 200 μl, Pierce Chemical Co., Rockford, Illinois, USA) were added and the stoppered vials treated for 20 min at 50°C. One microlitre of the sample was coinjected with 1 μl of cholesteryl-*n*-decylate (1.87 mg ml^{-1}) on to a Perkin Elmer Sigma 3B gas chromatograph fitted with a glass column (1 m×2 mm i.d.), packed with 3% OV1 on Diatomite CQ AW DMCS, 60–80 mesh (JJ's Chromatography, Kings Lynn) and flame ionisation detector. The

column temperature was 280°C, detector temperature 300°C and injector temperature 285°C. The carrier gas flow rate was 35 ml min^{-1}.

A calibration curve was constructed from injections equivalent to 0–25 μg soyasaponin I, hydrolysed and derivatised as in section 2.6. Upon hydrolysis a variety of sapogenols are produced (e.g. soyasapogenols B and C under the conditions used here). The relationship between x, the weight of soyasaponin I (before hydrolysis) and y, the summed peak areas of soyasapogenols B and C obtained by gas chromatography after hydrolysis was a straight line, linear fit $y=0.409x+0.061$, having a standard deviation of 0.361 and correlation coefficient of 0.997. When the original methanol extracts of soya flour were spiked with soyasaponin I at levels corresponding to 33, 83 and 167% of the estimated saponin content the recoveries were 94, 94 and 87%, respectively.

2.7. Mass spectrometry

The identities of soyasapogenols A, B and C were confirmed by direct comparison with authentic standards as well as by combined g.c.–m.s. using the trimethylsilyl ether derivatives. Gas chromatographic conditions were as listed in section 2.6. Electron impact mass spectra were recorded on a Kratos MS30 instrument, source temperature 250°C, nominal ionising voltage, 70 eV. The TMS derivative of soyasapogenol A (r.t. 26.9 min) showed characteristic ions at $^{m}/_{z}$ 762, 394, 379, 322, 291; that of soyasapogenol B (r.t. 18.4 min) at $^{m}/_{z}$ 674, 306, 291 and that of soyasapogenol C (r.t. 9.4 min) at $^{m}/_{z}$ 584 and 216.

3. Results and discussion

3.1. Lipid contents

The lipid contents of the samples examined in the present study are presented in Table 1. Also shown for comparison purposes are the lipid contents, where available, of the same species as quoted by Pattee *et al.*[10] In general the agreement is good and where differences are observed (chick pea and jack bean) this may be attributed in part to presumed differences in the varieties examined.

Table 1. Lipid and saponin contents[a] of legume seeds, peanut and etiolated bean shoot

	Sample	Percentage $CHCl_3$ solubles	Percentage saponin content[b] g.kg^{-1}	Literature[c]
1	Soya	21.0	6.5	(54)
2	Mung	2.0	0.5	(6.1)
3	Bean shoot	0.3[d]	1.1[d]	
4	Green pea	3.0	1.8	(11, fresh pea)
5	Yellow split pea	2.7	1.1	
6	Lentil	2.0	1.1	(5.1)
7	Chick pea	8.2	2.3	(61)
8	Peanut	46.7	<0.1	(11)
9	Runner bean	2.7	3.4	
10	Kidney bean	2.4	3.5	(16)
11	Butter bean	1.0	1.0	
12	Haricot bean	2.3	4.1	(19)
13	Field bean	2.4	0.1	(3)
14	Jack bean	3.7	n.d.	

[a] Expressed on a defatted flour basis.
[b] Assumes a sugar:sapogenin ration of 1:1.
[c] Reference 13, expressed on a defatted flour basis.
[d] Expressed on a dry weight basis, shoots contained 93% water.

3.2. Saponins

3.2.1. Quantitative analysis

The saponin contents of the 15 samples examined in the present study are shown in Table 1, only jack bean (sample 14) being free of these compounds. Saponins are expressed as a weight percentage of the defatted flour, assuming that the aglycone:carbohydrate ratio is 1.0. However this can only be an approximation, for example in soya the five reported saponins[9] (I, II, III, A1 and A2) have aglycone:carbohydrate ratios of 0.9:1, 1:1, 1.3:1, 0.6:1 and 0.7:1 respectively while their relative composition in the saponin mixture present in soya has been reported[5] to be 60, 6, 1, 30 and 3%, respectively. Other workers have reported more complex saponins in *P. vulgaris*[11,12] which will further reduce this ratio. Thus before a more accurate saponin content can be assigned, information is needed about the chemical nature of the saponins present in the sample, see below.

For comparison purposes the saponin contents, where available, reported by Fenwick and Oakenfull[13] for these products are given. The range found in the present study (0–0.61%) is much less than that found by the Australian workers[13] (0.3–6.1%), even when the latter are calculated on the basis of the same lipid contents as presented in Table 1. Suggestions have been advanced elsewhere[6,9] to explain the reasons for such discrepancies—notably the incorrect assignment[13] of several major components on the thin-layer chromatogram as saponins on the basis of a relatively non-specific spray reagent.

Examination of the literature reveals few comparable data. Recently the saponin contents of *P. vulgaris* beans grown in Nigeria and peanuts have been reported to be 0.024% and 0.005%, respectively,[14] but few details were presented by the authors as to the method of quantitation used. A more extensive study was conducted by Livingstone *et al.*,[15] who used an assay based upon the activity of saponins in a fungal bioassay using a *Trichoderma* species although this assay is only appropriate for the acidic saponins of alfalfa (e.g. possessing hederagenin—and medicagenic acid aglycones) and is insensitive to the neutral soyasaponins. The results obtained by these workers (frozen green beans, 0.05% dry weight; red beans, 0.02%; dried peas, 0.03%; dried black-eyed peas, 0.03%; dried soya beans, 0.01%; bean sprouts, 0.01%; frozen green peas, 0.01% and snow peas, 0.01%) should be viewed in this context. One interesting finding is that alfalfa sprouts contain very high (~8%) levels of acidic saponins[15] and, given the biological activity of these compounds and the increased human consumption of bean sprouts, further work is considered necessary in this area. It will be seen, however, (Table 1), that the commercial bean shoots examined in the present study (sample 3, not alfalfa) contained only low levels of the biologically less active soyasaponins.

Recent determinations of the saponin content of soya bean meals range between 0.3–0.4%,[5,7] somewhat lower than the figure shown in Table 1. In order to investigate this in more detail soya bean meal, sample 1, was subjected to the analytical procedures of Kitagawa *et al.*[9] and Ireland and Dziedzic.[7] The former method, which produced a figure of 0.48%, uses saponins as internal standards; however losses (~10%) were found to occur in the preliminary sample clean-up step involving butanol–water partition. These losses do not occur in the flash chromatography clean-up stage reported here. The latter method, which uses soyasapogenols as standards, gave a figure of 0.46%. In the present work hydrolysis of isolated soyasaponins were studied and recoveries of 94% for soya extracts spiked with soyasaponins as mentioned in section 2.6 were obtained. However the recovery of soyasapogenols from soyasaponins is much lower. The procedure adopted by Ireland and Dziedzic[7] uses a different hydrolysis medium to that described here, which has been observed to produce soyasapogenol E as an artefact, and possibly will also give a different yield of soyasapogenol from the parent, intact saponins. However, more importantly the method was calibrated against soyasapogenols which therefore assumes a molar relationship between glycoside and aglycone. In our experience this is not necessarily the case, and would expect the true saponin figure to be higher than that found.

3.2.2. Qualitative analysis

Inspection of the various thin layer chromatograms, Figure 1, reveals the complexity of the saponin areas which undoubtedly explains the problems associated with using this method for quantita-
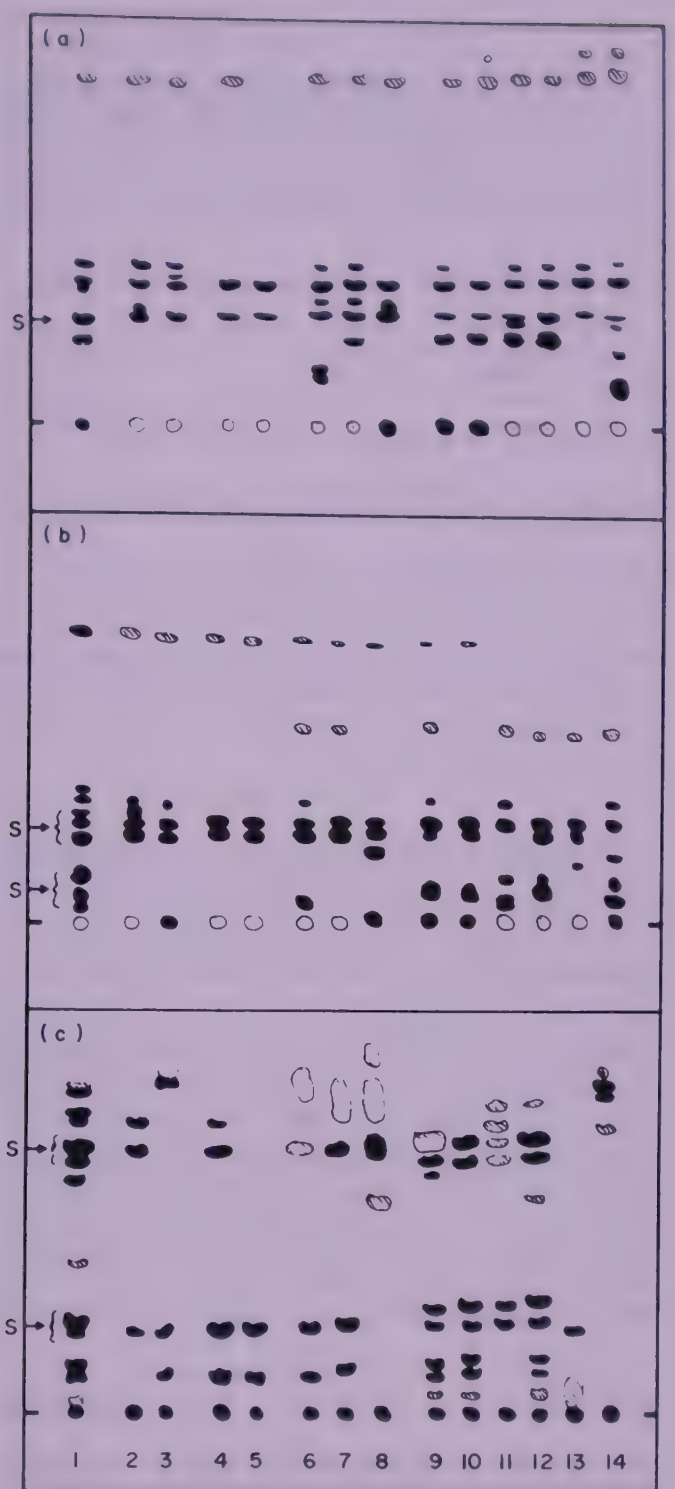
Figure 1. Thin-layer chromatograms of individual saponins in sample extracts. (a) Silica gel plate; solvent system, 1-butanol:ethanol:0.880 ammonia (7:2:5). (b) Silica gel:oxalic acid plate; solvent system, chloroform:methanol:water (65:35:10; lower phase). (c) Reversed phase (C_{18}) silica gel plate; solvent system, methanol:water (3:2). Numbering of samples as in Table 1, S indicates the position of the saponins.

tion.[6,13] Figure 1 shows the separation in the three t.l.c. systems used of individual saponin species for all 14 samples. In Figure 1a, it can be seen that on silica gel all the saponins run as a single spot (S), having R_F 0.26. The use of silica gel-oxalic acid[9] (Figure 1b) and reversed phase C_{18} silica gel plates (Figure 1c) allows good separation of the component saponins, this being especially clear in the latter example where the monodesmosidic saponins[17] (I–III) show R_F~0.22 whilst the bisdesmosidic saponins (A1 and A2) are widely separated from these, having R_F~0.65. Such separations of the different types of saponin have been further exploited by the use of flash chromatography to effect separation of relatively large amounts of these individual compounds (Price, K. R., unpublished). Thus the use of t.l.c. (Figure 1, b and c) may be seen to provide information about the nature of the individual saponins present. For instance in soya bean (sample 1) the separation of mono- and bisdesmosidic saponins, possessing one and two carbohydrate chains respectively, is clearly shown.

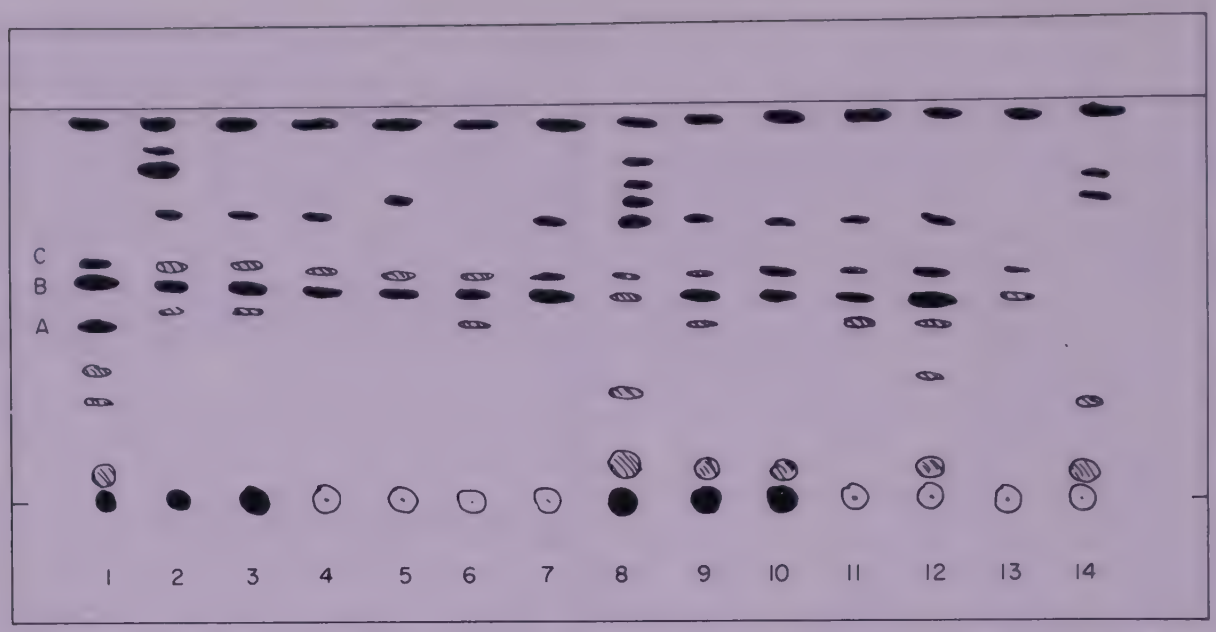

Figure 2. Thin-layer chromatogram of the hydrolysates of sample extracts showing the individual sapogenols (A, B and C). High performance silica plate: solvent system, chloroform:methanol (96:4), numbering of samples as in Table 1.

In Figure 2 the high-performance thin-layer chromatogram of the sapogenols released after acid hydrolysis is shown. Soyasapogenols A, B and C are all well separated and can be seen to be present in soya (sample 1); in most samples however only soyasapogenols B and C are observed, in agreement with the results obtained by gas chromatography. In the course of the qualitative and quantitative studies reported here a linear relationship was established between the levels of soyasapogenols B and C, occurring as a result of acid hydrolysis, over the range of saponin contents (0–0.65%) encountered.[16] This is strongly suggestive of the latter being an artefact of hydrolysis rather than a natural product, in agreement with observations by others.[18–20] The use of enzymic, rather than chemical, hydrolysis would be expected to clarify the situation.

It is noteworthy that only trace amounts of soyasaponin I (and soyasapogenols B and C) were found in the peanut (sample 8), although this compound has recently been reported in the roots of the plant[21] and some evidence has been presented to suggest that it may occur in peanut hearts and thereby cause problems of excessive bitterness.[22,23]

4. Conclusions

The analysis of saponins is complex and potentially subject to considerable errors during the isolation, separation and quantitation stages. For these reasons many of the early reports of saponin contents of food plants and processed foods must be treated with circumspection. The techniques used in this paper, involving qualitative and quantitative measurements, are specific for saponins and are considered to provide a more reliable figure. It is acknowledged that a method based upon separation and quantitation of the individual saponin species present (e.g. using h.p.l.c.) would be highly desirable. Thus while the general nature of the saponins (e.g. mono or bisdesmosides) and the structure of the aglycone are known from the data presented here, the exact structures of the saponins are unclear which means that the contents quoted are only approximate. Whilst it is probable that the compounds correspond to some, or all, of the five saponins isolated and characterised in soya (soyasaponins I, II, III, A1, A2)[5] the occurrence of additional, related, compounds cannot be excluded. At present studies are continuing to develop a rapid screening method to identify such components in crude plant extracts and preliminary results suggest that mass spectrometry using fast atom bombardment may be especially useful in this respect (Price K. R.; Eagles, J.; Fenwick, G. R., unpublished).

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J. Sci. Food Agric. 1986, **37**, 1192–1196

A Small, Simple Sorghum Decorticating Device

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A small commercially available laboratory mill was found to be suitable to dehull small batches of sorghum grain. The device used one grinding plate and an agitating dimpled plate and took about 1 min to adequately dehull the grain. At the completion of dehulling period decorticated grain was collected directly in a sample holder. Data from various cultivars, grain sizes and types of grain were reproducible. It only required 10 s to clear the device between samples.

Keywords: Sorghum; grain; dehuller; hardness.

1. Introduction

Substantial developments are being made in the sorghum industry in developing countries. Traditional, low-yielding varieties are being replaced with high-yielding hybrids from developed countries, active breeding and agronomical programmes have been initiated and traditional manual milling is being replaced by industrial mills with sorghum flour now commercially available.[1–6]

Dehulling is essential for consumption of sorghum but dehulling characteristics vary enormously between cultivars[7–9] so there is need for an objective measure of dehulling qualities of breeding lines at an early stage of selection and also on commercial grain. A few instruments are available to measure dehulling characteristics[10,11] but they are cumbersome to use and require close attention to detail to obtain reproducible results.

The present study describes the application of a simple device that can be used to measure decortication characteristics of small batches of sorghum.

2. Experimental

2.1. Description of dehuller

The dehuller used (Figure 1) was a small commercially available unit (Pearlest, Kett Electric Laboratory, Japan) of overall dimension $16 \times 9.5 \times 13$ cm^3. It consisted of a fixed corrugated metal abrasion disc (a) which fitted into a hinged lid which when closed abraded against a dimpled agitating plate (b). After the dehulling period, lever (c) is opened and the dehulled sample passes into sample holder (d) which comprises a 20 mesh sieve. The abraded fraction is swept through a narrow space around the agitating plate during dehulling by a horizontal moving metal bar (e) fitted beneath the agitating plate and collected.

2.2. Dehuller operation

A sorghum sample (5 g) was spread over the agitating plate, the lid closed and the dehuller run for a specific time. The lever was opened to release the dehulled sample into the holder which was shaken thoroughly to allow the smaller particles to pass through and the abrasion plate was brushed to remove adhering fine powder. The machine was then ready for the next run.

The time needed for the required dehulling level was termed as hardness; the weight retained in

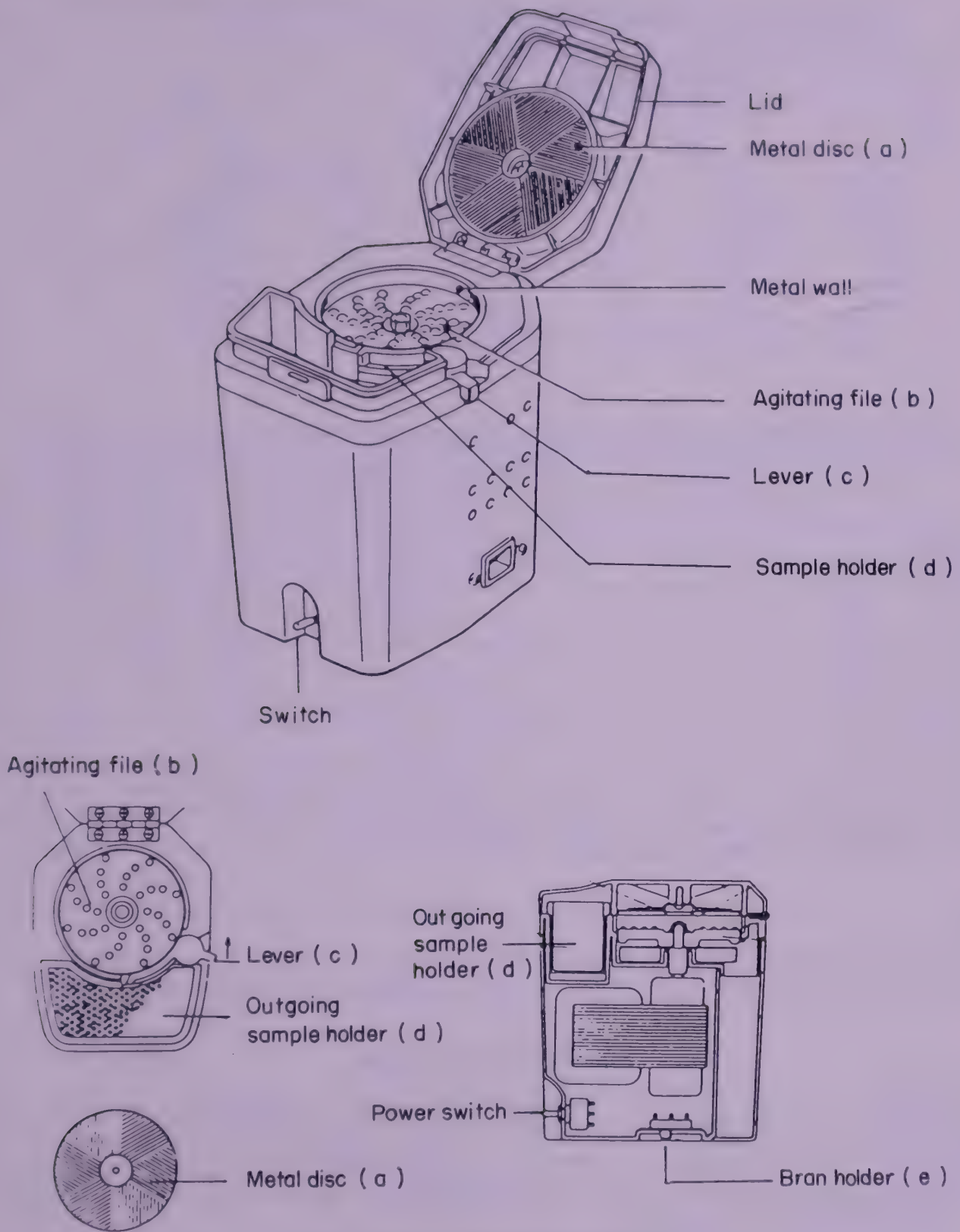

Figure 1. The Kett Husk Peeler, 'Pearlest'.

the holder as milling yield and the loss of weight as milling loss (ML). Dehulled unbroken kernels with germ attached were separated manually from broken kernels and weighed separately.

2.3. Experimental studies

Milling performance of various sorghum cultivars was assessed by dehulling both to a fixed milling loss (usually 10%) and for a fixed time (60 s). Abrasive hardness (AHI) was calculated as the time in seconds for each per cent of milling loss.[11]

Table 1. Standard deviation and coefficient of variation of decortication of Sorghum grain (3.35–3.99 mm size)

	Dehulling to 10% milling loss				Dehulling for 60 s	
	Sound		Damage		Sound	
Cultivar	ML[a]	c.v. (%)	ML[a]	c.v. (%)	ML[a]	c.v. (%)
Dorado	10.2±0.28	2.82	9.5±0.49	5.22	11.2±0.30	2.70
SM8	9.8±0.20	2.53	10.1±0.42	4.15	12.5±0.55	4.45
C43	9.4±0.22	2.09	9.9±0.59	5.98	13.2±0.45	3.41
Goldrush	9.7±0.14	1.50	9.8±0.38	3.66	15.0±0.60	4.10
E57	9.9±0.35	3.58	10.0±0.49	4.89	17.0±0.54	3.19

[a]Mean±standard deviation of the mean of ten replicates.

Table 2. Dehulling time and milling loss of sound and weather-damaged sorghum grain of different sizes

	Dehulling to 10% milling loss (3.35–3.99 mm)						Dehulling for 60 s (different sizes)					
	Sound grain			Damaged grain			>4.00 mm		3.35–3.99 mm		2.80–3.35 mm	
Cultivar	MT	ML	AHI	MT	ML	AHI	ML	AHI	ML	AHI	ML	AHI
Dorado	57 (55.3)	10.3	5.5	36	9.8	3.7	12.8	4.7	11.4	5.3	9.9	6.1
SM8	48 (51.1)	9.4	5.1	45	10.1	4.4	—	—	12.5	4.8	11.2	5.4
C43	50 (51.0)	9.8	5.1	39	9.9	3.9	15.1	4.1	13.2	4.5	10.5	5.7
Goldrush	46 (46.9)	9.9	4.7	42	10.1	4.5	15.7	3.8	14.0	4.3	11.3	5.3
E57	35 (35.4)	9.9	3.5	31	10.0	3.1	22.3	2.7	17.7	3.4	15.1	4.0

MT, milling time; ML, milling loss; AHI abrasive hardness index =MT/ML.
Figures in parentheses adjusted to 10% milling loss.

Sequential dehulling of Dorado and SMB cultivars was carried out with up to 50% milling loss. Colour reflectance of ground dehulled kernels was measured as described by Ali and Wills.[12]

3. Results and discussion

The dehuller was used to decorticate ten replicates of 5 g samples of size-graded sound and weather damaged grain for a fixed time that gave 10% milling loss and for 60 s (sound grain only). The standard deviation and coefficient of variation (c.v.) presented in Table 1 show that the extent of decortication was reproducible for all cultivars and both types of grain although the c.v. was lower for sound grain than damaged grain.

The dehulling characteristics are presented in Table 2. The cultivars showed the same order of hardness in milling regardless of whether milling loss (ML), milling time (MT) or AHI were used as the milling index. The MT of the damaged grain was lower than that of the sound grain and MT could be used to differentiate between sound and damaged grain. The ML decreased with decreasing size of grain and consequently AHI increased; thus evaluation of a sorghum cultivar by milling without size-grading could give erroneous conclusions. While grain hardness can be expressed in terms of MT and ML dehulling for fixed time (i.e. using ML) is easier and quicker for evaluation of cultivars.

Separation of the dehulled products into broken and unbroken kernels showed an average of 85% unbroken kernels were obtained indicating that the device is highly efficient in dehulling. Reports on recovery of unbroken kernels using other dehulling devices have been by Shepherd[10] who obtained

Table 3. Correlation coefficients of sequentially dehulled Dorado and SM8 sorghum grain

	Milling time	Milling loss	Unbroken kernel
Dorado			
Colour	−0.77**	−0.84***	0.87***
Unbroken kernel	−0.96***	−0.99***	
Milling loss	0.99***		
SM8			
Colour	−0.91***	−0.93***	0.93***
Unbroken kernel	−0.99***	−0.99***	
Milling loss	0.99***		

**$P<0.01$; ***$P<0.001$.

77% unbroken kernel from a single cultivar and De Francisco *et al.*[13] who reported 68% of intact decorticated grain including degermed unbroken kernel. However, it is recognised that the performance of any dehuller is markedly dependent on the grain characteristics.[9,12]

The relationship between the different fractions of sequentially dehulled grains of Dorado and SM8 cultivars are presented in Table 3. In both cultivars MT was significantly correlated with ML, the level of unbroken kernels and the colour reflectance value of unbroken kernels. MT and ML were inversely correlated to the yield of unbroken kernel and colour reflectance value. These are in agreement with data of Reichert *et al.*[14] but the calculation of AHI (251.2 for Dorado and 228.3 for SM8) was quite different from the indices determined from 10% milling loss and milling loss after 60 s. Figures 2 and 3 show that milling loss and brokens increased as MT increased and unbroken kernel decreased. Dehulling up to 15% ML, the MT and ML were similar in both cultivars but the extent of broken kernels then increased. Shepherd[10] recorded that ML initially increased rapidly then levelled off but the opposite phenomenon was observed for broken kernels. Ooomah *et al.*[11] reported a difference in milling behaviour between soft and hard grain and De Francisco *et al.*[13] showed differences in dehulling patterns of three cultivars. Thus, differences in dehulling behaviour exist between cultivars.

The operation of the described dehulling device was simple, convenient and rapid with a time

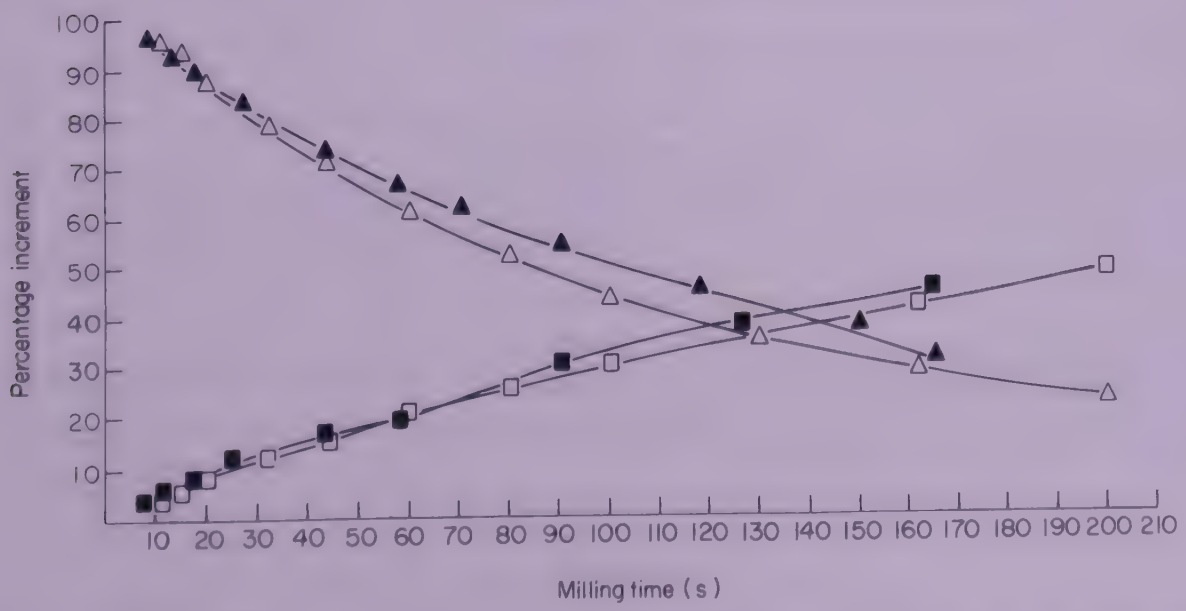

Figure 2. Sequential dehulling of Dorado and SM8—milling time and unbroken kernel and milling loss: (△), unbroken kernel in Dorado; (▲), unbroken kernel in SM8; (□), milling loss in Dorado; (■), milling loss in SM8.

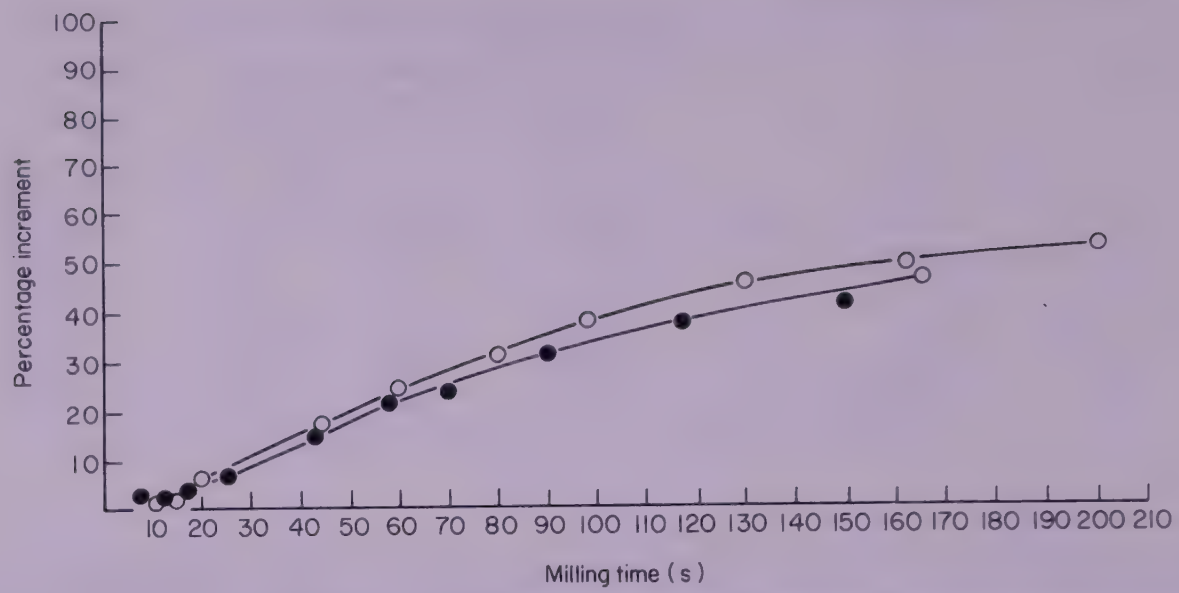

Figure 3. Sequential dehulling of Dorado and SM8—milling time and brokens: (○), brokens in Dorado; (●), brokens in SM8.

delay of about 10 s to clear the machine between samples. Both large and small grains can be dehulled separately with this dehuller without adjustment. The machine has also been used successfully for pearling barley and rice.

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Nutritive Value of Rapeseed Meal: Effects of Individual Glucosinolates

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In the first of a series of studies on the nutritional value of rapeseed meal, rats were fed diets containing six individual glucosinolates over a period of 29 days. The diets included progoitrin and gluconapin both of which are major components of high glucosinolate rapeseed cultivars. No reduction of feed intake or growth rate was noted and thyroid weights were significantly affected only by the progoitrin diet, which also produced small increases in the weights of the livers and kidneys. There was no depression of plasma thyroid hormone levels.

Keywords: Rapeseed; feeding trials; nutrition; glucosinolates; thyroid; rats.

1. Introduction

Rapeseed is a crop of considerable economic importance in Europe. Production in the EEC is currently 3.6 Mt, approximately 20% of the global figure. The main producing countries are France (1.1 Mt estimated for 1985–1986), United Kingdom, W. Germany (710 000 t) and Denmark (370 000 t).[1] In the UK, production in 1985–1986 was originally estimated to exceed 900 000 t,[1] however unfavourable growing conditions make it likely that the figure will be nearer 800 000 t.

Although grown primarily for its high oil content (40–45% of the seed) rapeseed provides a defatted meal which has been used as a source of protein for animals and poultry. Such use is limited by the presence of antinutritional components, e.g. glucosinolates, tannins and fibre—amongst which the first are generally regarded as the most important.[2] Although plant breeders have reduced the levels of glucosinolates in some cultivars (notably those grown in Canada and Denmark) to below 20 μmol g^{-1} seed, for most of the European crop the levels are much higher, typically above 40 μmol g^{-1} seed. The inclusion of rapeseed (and *Crambe abyssinica*) meals containing high levels of glucosinolates in animal feeds has resulted in problems of reduced feed intake, enlarged thyroids, reduced levels of circulating thyroid hormones and, occasionally, has been linked to more serious disorders, including organ abnormalities and death.[2–5]

There have been only a few studies conducted into the metabolism and biological activity of individual glucosinolates in the mouse,[6] chicken[7] and rat.[8–10] Until recently the main barrier to such investigations has been the lack of availability of such compounds in sufficient amount and purity to facilitate meaningful animal experimentation. Within the framework of the research agreements between INRA (Institut National de la Recherches Agronomique) and CETIOM (Centre Technique Interprofessionnel des Oléagineux Métropolitains) a study of the nutritional effects of glucosinolates, with especial emphasis on the major glucosinolates in rapeseed, was deemed important. A collaborative study between groups at the INRA, Centre de Recherches Zootechniques et Veterinaires, and AFRC Food Research Institute, Norwich was therefore initiated and the present paper contains the initial results of this work.

2. Experiment

2.1. Animals

Sprague Dawley male rats, each weighing 70 g, were randomly divided into seven groups of eight animals.

2.2. Glucosinolates

The glucosinolates examined in this study were gluconapin (but-3-enyl glucosinolate), and progoitrin (2-hydroxybut-3-enyl glucosinolate) which occur in rapeseed; glucobrassicin (3-indolylmethyl glucosinolate) which is considered typical of the indole glucosinolates in rapeseed, sinigrin (prop-2-enyl glucosinolate) and sinalbin (*p*-hydroxybenzyl glucosinolate), the last two of which may contaminate commercial rapeseed rations as a result of the presence of mustard (*B. juncea*) and stinkweed (*Thlaspi arvensis*) or wild mustard (*Sinapis arvensis*), respectively. In addition a sample of glucotropaeolin (benzyl glucosinolate) was also included in the study. The individual glucosinolates (1–10 g amounts) were isolated and purified according to published procedures[11, 12] using the following sources (seed, unless specified to the contrary): sinigrin (*Brassica nigra*), gluconapin (*Brassica campestris*, rapesed, cv. Sarson), progoitrin (*Brassica napus*, swede, cv. Laurentian), glucosinalbin (*Sinapis alba*), glucobrassicin (young leaves of Brussels sprout, *Brassica oleracea*), glucotropaeolin (*Lepidium sativum*). The purities of the isolated glucosinolates were assessed by glucose release[13] and gas chromatography[14] to be >99% (sinigrin, glucobrassicin, glucotropaeolin and sinalbin) and >90% (gluconapin). Despite repeated attempts at further purification the isolated progoitrin, although homogeneous in glucosinolate content, was only ~80% pure as determined by glucose release.

2.3. Experimental diets

The control diet comprised herring meal (150 g) supplemented with methionine (2.5 g) to meet amino acid requirements, wheat starch (769.5 g), maize oil (20 g), minerals (40 g) and a vitamin mixture (18 g kg^{-1} dry matter). It was prepared in powder form. Previous experiments had demonstrated daily growth rates of 7–7.5 g in rats fed this diet.

The six glucosinolate-containing diets each consisted of the control diet supplemented with one glucosinolate. The amount of each glucosinolate added was determined by the availability of the material and, in the case of progoitrin, by the relative amounts found in rapeseed meal. Progoitrin was included at a level of 3 g kg^{-1}, sinigrin, gluconapin, glucosinalbin and glucotropaeolin at 1 g kg^{-1} and glucobrassicin at 0.5 g kg^{-1}.

The rations were prepared daily by moistening the powdered control diet and adding the requisite amount of glucosinolate dissolved in distilled water (5 ml). Thereafter the diets were thoroughly mixed and fed *ad libitum*. Analysis revealed all glucosinolates to be stable under these conditions for the period of the feeding trial. The rats were adapted progressively to the diets as follows: 2 days on the control diet, 1 day on control diet containing 50% of the final glucosinolate level, 1 day on control diet containing 75% of the final glucosinolate level. Thereafter the glucosinolate containing diets, as above, were fed for 29 days. At the end of this period, the animals were anaesthetised with diethyl ether for blood sampling and sacrificed by intracardial puncture.

2. 4. Methods and analysis

The amount of diet consumed daily was noted and cumulated throughout the whole experiment. Animals were weighed on Mondays, Wednesdays and Fridays to determine growth rate. Appearance (activity, behaviour, state of fur) was noted daily.

Upon slaughter, portal and peripheral blood samples were taken for analysis. Thyroid, liver and kidneys were excised and weighed and the former stored for histopathological examination. The contents of the digestive tract, small intestine and caecum were removed and the remaining carcass weighed. Plasma thyroid hormone levels were determined by radio-immunoassay as described by Davicco *et al.*[15]

Table 1. Effects of glucosinolates in the growing rat

	Diets							Least significant difference ($P<0.05$)
	Control*	(3 g kg^{-1}) Progoitrin	(1 g kg^{-1}) Sinigrin	(1 g kg^{-1}) Gluco-napin	(1 g kg^{-1}) Sinalbin	(1 g kg^{-1}) Gluco-tropaeolin	(0.5 g kg^{-1}) Gluco-brassicin	
Dry matter (g day^{-1})	18.81^{a} ±1.94	19.90^{ab} ±1.12	20.24^{b} ±1.43	19.92^{ab} ±1.16	19.67^{ab} ±1.08	20.27^{b} ±1.19	19.58^{ab} ±1.80	1.43
Live weight gain (g day^{-1})	7.13^{a} ±0.55	7.43^{a} ±0.86	7.39^{a} ±0.75	7.54^{a} ±0.56	7.48^{a} ±0.81	7.38^{a} ±0.83	7.18^{a} ±0.84	0.75
Live weight gain empty (g day^{-1})	6.83 ±0.49	7.06 ±0.85	7.01 ±0.67	7.22 ±0.06	7.06 ±0.74	7.12 ±0.84	6.93 ±0.85	0.73
Thyroid weight (mg rat^{-1})	13.5 ±1.2	18.1 ±2.7	15.3 ±2.1	16.1 ±1.7	13.5 ±1.6	16.2 ±2.3	14.6 ±2.4	2.1
Weight (mg 100 g^{-1}) live weight empty	5.33^{a} ±0.38	6.70 ±1.65	5.60^{a} ±0.82	5.75^{a} ±0.68	4.90^{a} ±0.72	5.86^{a} ±0.85	5.38^{a} ±0.86	0.92
Liver weight (g rat^{-1})	15.32 ±1.66	17.92 ±2.24	16.58 ±1.82	17.08 ±2.53	16.14 ±2.16	14.79 ±1.89	14.41 ±1.89	2.02
Weight (mg 100 g^{-1}) live weight empty	5.66^{a} ±0.30	6.50^{c} ±0.43	6.04^{b} ±0.35	6.06^{b} ±0.56	5.81^{ab} ±0.45	5.31^{a} ±0.31	5.29^{a} ±0.38	0.41
Kidneys weight (g rat^{-1})	2.09 ±0.18	2.28 ±0.31	2.12 ±0.21	2.16 ±0.25	2.12 ±0.16	2.09 ±0.18	1.98 ±0.14	0.21
Weight (mg 100 g^{-1}) live weight empty	783^{a} ±48	828^{ab} ±77	770^{ab} ±54	765^{a} ±56	770^{ab} ±72	754^{a} ±43	731^{a} ±53	59
Thyroid hormones T_3 (pg ml^{-1} plasma)	640 ±120	560 ±120	690 ±120	720 ±210	730 ±80	710 ±170	630 ±150	NS
T_3 (ng ml^{-1} plasma)	40.4^{ac} ±5.0	38.4^{a} ±4.8	51.4^{bc} ±6.7	49.7^{c} ±12.0	43.9^{abc} ±4.2	39.9^{a} ±3.6	47.6^{abc} ±17.2	0.94

^{abc} Values with different superscripts within a row are significantly different; $P<0.05$.

3. Results

The results are presented in Table 1. Although the quantities of feed ingested were very high (equivalent to an average of 20 g dry matter day^{-1}) there was an unusual individual variability, especially in the control group. All of the glucosinolate-containing diets were consumed in greater amounts than the control, with those containing sinigrin and glucotropaeolin being significant ($P<0.05$). No depressive effect was noted at the end of the experiment.

The rats exhibited a regular and high growth rate (7.4 g day^{-1} on average) on all of the diets. Ingestion of purified glucosinolates not only had no depressive effect on growth rate, but a small (though non-significant) increase was noted and was maintained until the end of the experiment.

Thyroid weight was significantly affected only by the diet containing progoitrin, an average increase of 26% being observed ($P<0.01$). The increase ranged from 0–95% and was inversely related to the live weight gains.

An increase of 15% ($P<0.01$) was observed in the weight of the livers in the group fed the progoitrin-containing diet. Diets containing sinigrin and gluconapin also produced increases (+7%, $P<0.05$) but no other diets had significant effects. The weight of the kidneys was increased (7%, $P<0.05$) in the group fed progoitrin but was not significantly affected by any of the other glucosinolates.

None of the glucosinolate-containing diets depressed plasma T_3 or T_4 levels. In the groups fed sinigrin and gluconapin, plasma T_4 levels were significantly higher (27%, 23% respectively, $P<0.05$) than in the control group. No such effect was noted on plasma T_3.

4. Discussion

In the present study the inclusion of sinalbin in the diet at 1 g kg^{-1} produced no adverse effects. In an early study, Josefsson and Uppström[6] fed much higher levels (thirty to sixty-fold) to mice for 11 days and observed severe reductions in weight gain (−75%), feed intake (−38%) and PER (protein efficiency ratio) (−55%). In agreement with the present findings, Bille *et al.*[10] observed no adverse affects on feed consumption, weight gain or true digestibility to be associated with the feeding of 0.2, 1 or 5 g sinalbin kg^{-1} diet. However these workers found that BV (biological value) and NPU (net protein utilisation) were both negatively affected at the highest inclusion level.

As part of a particularly detailed investigation, Nishie and Daxenbichler[8] found that subcutaneous injection of sinalbin (300 mg kg^{-1} body weight on days 8 and 9 of gestation) had no effect on subsequent weight gain but did increase the relative liver weight of the pregnant rat. The same workers administered sinigrin both orally and subcutaneously on days 8 and 9 of gestation (322 mg kg^{-1} body weight). No effect on weight gain was observed and only for the group injected with sinigrin was there an associated increase in liver weight. However, oral administration of sinigrin increased the thyroid weight in pregnant and non-pregnant rats. Bille *et al.*[10] similarly found thyroid weights to be increased following a daily level of 5 g sinigrin kg^{-1} (ration) and liver weight was again unaffected. Palatability and weight gain were also adversely affected at this level of inclusion. In the present experiment, sinigrin and the higher homologue, gluconapin, both increased liver weights over the 29 day period of the experiment. These two glucosinolates also caused an increase (>20%) in plasma T_4 levels. The goitrogenic effect of sinigrin may be mediated *via* prop-2-enyl isothiocyanate,[16] which is metabolised to SCN^-.

The antithyroid effect of 5-vinyloxazolidine-2-thione, formed following initial enzymic deglycosylation of progoitrin has been known from feeding rats using rapeseed and *Crambe abyssinica*.[2, 5] Bille *et al.*[10] observed palatability to be adversely affected with a daily dose of 5 g kg^{-1}. Liver, kidney and thyroid weights were all increased as dietary progoitrin increased. In the present study, progoitrin at levels of 3 g kg^{-1} increased liver and thyroid weights but growth was not depressed. Epi-progoitrin (the epimer of progoitrin) has been found to have no effect on weight gain of pregnant rats following the subcutaneous injection of 159 or 320 mg kg^{-1} on days 8 and 9 of gestation.[8] When added to the diet of laying hens (230 mg kg^{-1} diet), progoitrin caused inhibition of hepatic-TMA oxidase, a property of oxazolidine-2-thiones and related thioamides. When tested *in*

vitro, neither progoitrin nor gluconapin caused such inhibition, suggesting that metabolic processes were needed to generate active principles.[17]

It is tempting to rationalise the various biological effects associated with dietary progoitrin on the basis of metabolic conversion to known active principles such as 5-vinyloxazolidine-2-thione,[3, 8, 17] 1-cyano-2-hydroxybut-3-ene[3, 8] and 1-cyano-2-hydroxy-3,4-epithiobutane[3, 8, 18, 19] and there is some direct evidence to support such a hypothesis. Smith and Campbell[20] have found hydroxynitriles to be produced more readily than oxazolidine-2-thiones in all areas of the digestive tract of hens fed rapeseed meal. Moreover a variety of breakdown products have been reported in relatively small amounts in the milk of animals fed rapeseed meal or forage brassicas,[21–23] although in the latter case the involvement of the plant myrosinase must be considered to be a distinct possibility. Lanzani *et al.*[24] have observed progoitrin when incubated with sheep rumen fluid to undergo significant polymerisation, presumably due to ring opening; in support of this mechanism traces of 5-vinyl-1, 3-thiazolidine-2-one were identified. Bille *et al.*[10] analysed the stomach contents of rats fed glucosinolates and found >90% of the intact compounds to have disappeared. No detectable amounts of glucosinolates were found upon analysis of faeces. In humans, Oginski *et al.*[25] have isolated a strain of bacteria capable of converting progoitrin to 5-vinyloxazolidine-2-thione. Recently a human faecal slurry has been shown to be capable of degrading sinigrin, progoitrin and glucobrassicin (N. Horn, J. Lewis, G. R. Fenwick, unpublished results) although the products have not yet been identified. However, in a study in which human volunteers consumed glucosinolate- (and, in particular, progoitrin-) rich cooked Brussels sprouts daily for 28 days,[26] no effect was observed on serum TSH, T_3 or T_4, suggesting the possibility that in the absence of myrosinase (inactivated by cooking) the extent of the breakdown of glucosinolates might be limited. Other possibilities, however, cannot be ruled out.

In the present study, neither glucotropaeolin nor glucobrassicin, showed any deleterious effects when fed at levels of 1 g and 0.5 g kg^{-1}, respectively. Whilst benzyl isothiocyanate, if formed in an analogous manner to prop-2-enyl isothiocyanate from sinigrin, might be envisaged to yield SCN^- and therefore exhibit an antithyroid effect, Goerler *et al.*[27] have recently shown orally-administered benzyl isothiocyanate to be metabolised in guinea pigs and rabbits to its cyclic mercaptopyruvate conjugate, 4-hydroxy-4-carboxy-3-benzylthiazolidin-2-thione.

Since indole glucosinolates are shown to be proportionately of greater significance in low—as compared with high glucosinolate rapeseed and it products, the inclusion of glucobrassicin in the present study is particularly important. From Table 1 it can be seen that no adverse effect is associated with its inclusion at levels comparable to those found in a diet containing 10% 'typical' low glucosinolate rapeseed meal. However it should be emphasised that, as with the other glucosinolates, behaviour in rats should not be uncritically extrapolated to livestock and poultry.

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Effects of Controlled Atmosphere on the Storage of New Apricot Cultivars

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The storage in refrigerated (0–0.5°C) and controlled atmospheres (5% O_2 and 0–7.5% CO_2) of fruit belonging to two new late-ripening cultivars of apricots (ICAPI-17-COL and ICAPI-30-COL) has been studied to determine their storability and to examine the effect of the CO_2 level on weight decrease, firmness, total titratable acidity (TTA), pH, refractometric degree (RD) and physiological and pathological changes. Both cultivars, but particularly ICAPI-30-COL, proved suitable to store for up to 3 weeks under controlled conditions. Only for ICAPI-30-COL did the development during storage of some ripening parameters (firmness, TTA, pH and RD) seem to be related to CO_2 concentration, showing the importance of varietal influence.

Keywords: Apricot; storage; controlled atmosphere; carbon dioxide; refrigeration.

1. Introduction

Italy is Europe's main producer of apricots (*Prunus armeniaca*), followed by Spain and Greece,[1] and production seems likely to rise considerably.

Not many data on the storage of apricots in refrigerated and controlled atmosphere (CA) are available in the literature, especially concerning the effect of CO_2 level in CA.[2–7]

At the University of Pisa, a programme of genetic improvement is under way to select new apricot cultivars of high commercial value and characterised by a late ripening time. They belong to a group of seedlings obtained by self-pollination at middle–late flowering and open-pollination of some previously obtained seedlings.[8]

The aim of this work was to examine the storage behaviour in CA of fruit belonging to two of the selected cultivars. Moreover the influence of CO_2 concentration on the development of weight decrease, firmness, total titratable acidity, pH, refractometric degree and physiological and pathological changes was studied.

2. Materials and methods

Apricots of cv. ICAPI-17-COL and ICAPI-30-COL were harvested at the colour changing stage, when the fruit skin had almost completely lost its green colour to reveal the typical yellow-orange colour of carotenoids. No pretreatments were carried out on the fruits.

The fruits (360 apricots for each cultivar) were immediately stored at 0–0.5°C in four different CA with CO_2 percentages of 0, 2.5, 5, 7.5 (±0.2) and an oxygen concentration of 5.0% (±0.2). A sample of each cultivar was air-stored at the same temperature for comparison.

The gas mixtures were previously saturated with water (r.h.=95–100% at 0–0.5°C) and the composition maintained constant during storage (24 days).

For each atmosphere tested, 72 fruits were initially stored and every 3 days a sample of six apricots was collected and their fresh weight, pH, firmness, total titratable acidity (TTA) and refractometric degree (RD) were evaluated.

The degree of physiological (over-ripening) and pathological degradation (spread of *Botrytis cinerea*) was given as a percentage of the fruits remaining in the storage cell.

An EFFEGI fruit-pressure tester equipped with a 0.79 cm plunger was used to measure the firmness of the pulp of the fruits which were peeled over selected small areas (three to four determinations for every fruit).

TTA was determined by diluting 10 g of juice with 100 ml deionised water and titrating to pH 8.0 with 0.05 M sodium hydroxide. Results were expressed as mEq 100 g^{-1} of fresh pulp. The other chemical analyses (RD, N, minerals, dry material and ascorbic acid) were carried out according to a standard method on the homogenised pulp maintained under nitrogen.[9]

3. Results and discussion

Table 1 reports some characteristics of the fruits belonging to the two apricot cultivars tested. The ripening time (7 and 20 days later than the Italian cultivar Cafona, as control) makes the two cultivars studied particularly interesting as, with correct storage, it is possible to keep the product on the market in a period (end of July to mid August) characterised by short supply. Size, shape, colour and firmness of the fruits make these two cultivars very promising commercially.

All the experimental data (Tables 2 and 3) have been correlated to the storage time using two different types of function: (a) linear for weight decrease, pH and firmness; (b) exponential for TTA and RD. The function coefficients (Tables 2 and 3) were calculated using the least squares method. In order to evaluate the possible linear correlation between the different variables with the CO_2 percentages of the atmospheres, the square of the correlation coefficient (r^2) between the value of the first derivative of the function (at the initial point in the case of the exponential curve) and CO_2 percentages was also calculated.

3.1. Weight decrease

The experimental data concerning the weight decrease of the fresh fruits are correlated to the storage time by a linear equation, cv. ICAPI-17-COL showed the largest effect. The CO_2 percentage in the storage atmosphere did not seem to effect weight decrease in either cultivars as shown in Figure 1a.

3.2. Firmness

For each cultivar a gradual linear reduction of the firmness was recorded. Figure 1b shows the curve for the angular coefficients (m) related to the correlation firmness/time, as a function of the increasing concentration of CO_2 in the atmosphere. The higher levels of CO_2 tended to maintain the

Table 1. Fruit characteristics of the two apricot cultivars tested

Characteristics	ICAPI-17-COL	ICAPI-30-COL
Ripening time (days after cv. Cafona)	7	20
Average weight (g)	43 (9.1)[a]	37 (8.3)[a]
Shape	Elliptical	Ovate
Skin colour	Light orange	Light orange
Skin overcolour	None	Suffused red areas
Pulp colour	Uniform light orange	Strong yellow
Firmness at ripening time (kg)	3.1 (6.3)[a]	3.2 (7.9)[a]
Edible portion (%)	94	91
Handling resistance	High	Middle–high
Dry material	12.0	14.0
Proteins (N×6.25) (%)	0.8	0.7
Minerals (%)	0.4	0.5
Ascorbic acid (parts×10^{-6}	100	110
Pulp pH	3.38	3.48
TTA (mEq 100 g^{-1} fresh pulp)	16.0	22.5
Refractometric degree	13.0	14.6

[a] v%. Standard deviation over mean×100.

Table 2. ICAPI-17-COL: weight decrease, firmness, TTA, pH and RD during storage

CO_2 (%)	Days of storage 3	6	9	12	15	18	21	24	r^2	$m.100$ or $k.100$
Weight decrease[a] *(%)*										
0	0.16	0.43	0.54	0.72	1.15	1.23	1.07	1.67	0.92	6.45
2.5	0.31	0.40	0.46	0.68	1.08	1.22	1.15	1.62	0.94	6.36
5	0.25	0.23	0.62	0.70	0.81	0.75	1.12	1.54	0.92	5.57
7.5	0.11	0.43	0.54	0.88	0.85	1.03	1.45	1.48	0.96	6.29
(air)	0.20	0.32	0.56	0.80	0.95	1.32	1.51	1.65	0.96	6.89
Firmness[a] *(kg)*										
0	2.9	2.6	2.4	2.5	2.3	2.3	1.9	1.7	0.92	−5.57
2.5	3.0	2.7	2.8	2.3	2.4	2.0	1.7	1.4	0.94	−6.29
5	3.0	2.9	2.6	2.7	2.6	2.5	2.4	2.3	0.94	−3.43
7.5	3.1	2.9	3.0	3.0	3.0	2.9	2.9	2.9	0.58	−0.95
(air)	2.9	2.7	2.4	2.3	2.5	2.0	1.9	1.4	0.90	−6.19
Total titratable acidity[b] *(mEq 100 g^{-1} fresh pulp)*										
0	14.0	12.4	10.7	9.5	8.3	9.6	6.5	5.5	0.92	4.11
2.5	14.9	13.7	12.4	10.7	10.5	9.0	8.5	7.5	0.96	3.07
5	15.1	14.0	13.7	13.0	12.2	11.6	10.2	10.4	0.94	1.88
7.5	15.7	14.9	14.6	15.0	14.3	14.3	13.4	13.7	0.88	0.72
(air)	13.4	11.0	9.7	7.5	6.7	5.0	5.5	3.9	0.96	5.81
pH[a]										
0	3.40	3.42	3.47	3.43	3.53	3.54	3.60	3.59	0.90	0.90
2.5	3.40	3.43	3.43	3.46	3.47	3.42	3.50	3.70	0.58	0.76
5	3.41	3.45	3.41	3.40	3.44	3.42	3.47	3.47	0.58	0.36
7.5	3.38	3.41	3.40	3.43	3.39	3.38	3.42	3.43	0.29	0.17
(air)	3.40	3.53	3.45	3.55	3.60	3.60	3.68	3.70	0.90	1.35
Refractometric degree[b]										
0	12.7	12.8	12.2	11.9	11.7	11.4	11.6	11.0	0.92	0.66
2.5	12.8	12.6	12.7	12.2	12.0	12.3	11.6	11.4	0.88	0.49
5	12.9	12.8	12.7	12.8	12.5	12.4	12.2	12.1	0.92	0.27
7.5	13.0	12.8	12.9	12.7	12.6	12.7	12.5	12.4	0.88	0.18
(air)	12.6	12.2	11.9	11.5	11.6	11.0	10.5	10.3	0.96	0.96

[a] Correlation function: $y=m.x+p$.

[b] Correlation function: $y=p.\exp(-k.x)$ where: y=quantity, x=days of storage, p=initial experimental value (see Table 1). For initial values, see Table 1.

firmness of the fruit tissues for ICAPI-17-COL, while ICAPI-30-COL was little affected by the CO_2 percentage.

3.3. Total titratable acidity (TTA)

For both cultivars TTA decreased during storage as shown in Tables 2 and 3. The rate of this drop was assumed to be directly proportional to the acidity concentration (Ac) and to the partial pressure of oxygen (PO_2) in the storage atmosphere, as shown by the following equation:

$$-dAc_{(t=t)}/dt=k_1.PO_{2(t=t)}.Ac_{(t=t)} \quad (1)$$

As PO_2 is constant in the experimental conditions, equation (1) reduces to:

$$-dAc_{(t=t)}/dt=k.Ac_{(t=t)} \quad (2)$$

where:

$$k=k_1.PO_{2(t=t)}$$

Integrating, the following equation is obtained:

$$Ac_{(t=t)}=Ac_{(t=0)}.\exp(-K.t) \quad (3)$$

Table 3. ICAPI-30-COL: weight decrease, firmness, TTA, pH and RD during storage

CO_2 (%)	Days of storage 3	6	9	12	15	18	21	24	r^2	$m \cdot 100$ or $k \cdot 100$
Weight decrease[a] (%)										
0	0.11	0.32	0.35	0.53	0.60	0.55	0.92	1.01	0.94	4.04
2.5	0.15	0.33	0.40	0.61	0.78	0.65	1.03	1.12	0.94	4.64
5	0.13	0.33	0.51	0.45	0.72	0.93	1.09	1.15	0.92	4.92
7.5	0.08	0.25	0.35	0.61	0.53	0.72	0.81	0.91	0.96	3.91
(air)	0.14	0.30	0.27	0.55	0.70	0.64	0.95	1.03	0.96	4.24
Firmness[a] *(kg)*										
0	3.0	3.1	2.9	2.8	2.8	2.6	2.7	2.4	0.88	−3.00
2.5	3.2	3.1	3.0	2.9	3.0	2.9	2.8	2.8	0.88	−1.76
5	3.2	3.1	3.1	3.0	2.9	2.9	2.8	2.7	0.96	−1.86
7.5	3.2	3.1	3.3	2.9	2.9	2.8	2.8	2.6	0.79	−2.06
(air)	3.1	2.9	3.0	2.8	2.8	2.1	2.4	2.2	0.82	−4.10
Total titratable acidity (mEq 100 g^{-1} fresh pulp)[b]										
0	21.0	16.4	17.2	15.1	13.0	11.6	10.6	9.7	0.84	3.56
2.5	20.9	16.4	16.4	15.0	15.2	12.5	11.5	9.7	0.92	3.30
5	20.9	19.8	16.7	13.6	13.7	12.4	11.0	10.2	0.86	3.37
7.5	20.6	19.4	15.1	15.0	13.0	13.3	11.3	10.3	0.92	2.46
(air)	18.1	11.5	12.0	10.3	8.2	6.5	4.5	4.0	0.94	7.21
pH[a]										
0	3.51	3.50	3.57	3.56	3.62	3.65	3.60	3.65	0.96	0.75
2.5	3.48	3.54	3.55	3.61	3.60	3.62	3.63	3.67	0.94	0.79
5	3.50	3.51	3.55	3.60	3.54	3.63	3.62	3.68	0.94	0.74
7.5	3.51	3.53	3.52	3.55	3.61	3.61	3.64	3.64	0.96	0.71
(air)	3.53	3.56	3.62	3.62	3.73	3.75	3.76	3.86	0.96	1.48
Refractometric degree[b]										
0	14.4	14.0	13.8	13.6	13.4	13.0	13.2	12.7	0.96	0.57
2.5	14.3	13.8	13.9	13.6	13.5	13.2	13.2	12.8	0.94	0.54
5	14.5	14.1	13.9	14.0	13.4	13.2	13.4	12.8	0.92	0.50
7.5	14.4	14.2	14.0	13.6	13.8	13.2	12.6	12.8	0.90	0.56
(air)	14.2	14.1	13.5	13.7	12.8	12.6	12.7	12.1	0.94	0.75

[a]Correlation function: y=m.x+p.

[b]Correlation function: $y=p.\exp(-k.x)$ where: y=quantity, x=days of storage, p=initial experimental value (see Table 1). For initial values, see Table 1.

Experimental data are well correlated by this equation, as shown by the values obtained for r^2. The variation of TTA in ICAPI-17-COL was affected by the CO_2 level in the CA (Figure 1c), decreasing as the CO_2 percentage increased. In the air-stored sample (high PO_2) this variation was much higher.

3.4. pH

Changes in pH with time in storage could be fitted to a linear equation (see r^2 values in Tables 2 and 3). A good correlation was also found between the angular coefficients for the curves of pH against time of ICAPI-17-COL and the PCO_2 in the atmosphere (Figure 1d). It is therefore possible to confirm that CO_2 tends to inhibit the disappearance of the acids in these fruits.

3.5. Refractometric degree (RD)

RD was taken as an indication of the amount of dry soluble substance, especially of sugars, that were present. As can be seen from Tables 2 and 3 there was a slight drop in the RD over time. This drop, caused by the respiration of the fruit, may be described, as for TTA, by an equation of the type:

$$RD_{(t=t)}=RD_{(t=0)}.\exp(-k.t) \quad (4)$$

A reduction in the rate of the RD drop over time in relation to the increase in the PCO_2 was also revealed for ICAPI-17-COL (Figure 1e), while ICAPI-30-COL did not seem to be affected, once more showing a difference between cultivars.

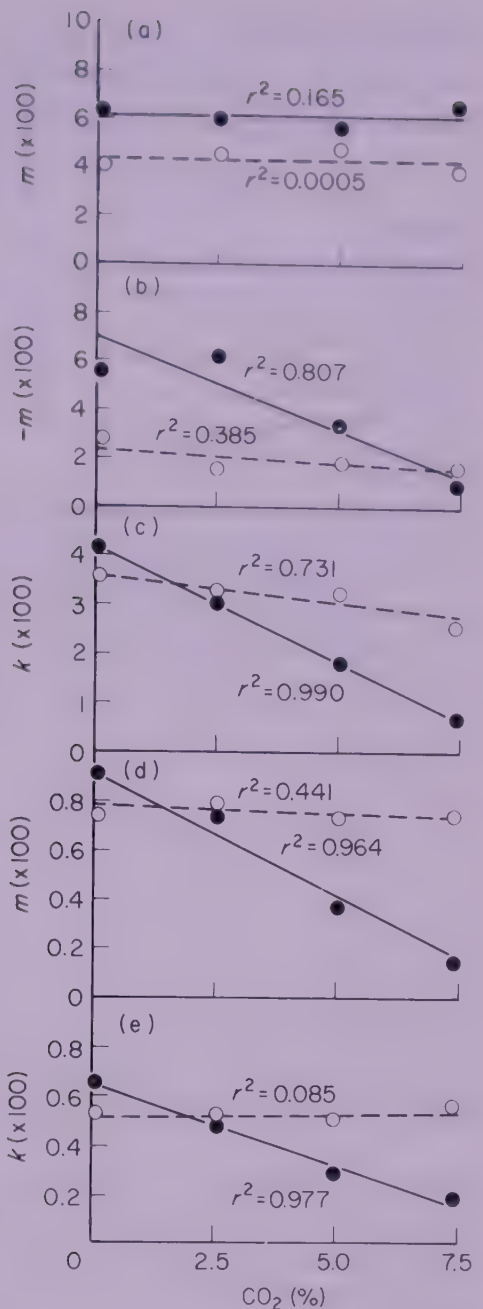

Figure 1. Influence of CO_2 concentration in CA on (a), weight decrease; (b), firmness; (c), total titratable acidity; (d), pH; (e), refractometric degree during storage of apricots, cv. ICAPI-17-COL (●—●) and cv. ICAPI-30-COL (○—○).

3.6. Physiological and pathological changes

ICAPI-17-COL, unlike ICAPI-30-COL, succumbed to attack by *Botritis cinerea* after 18 days, and by the twenty-fourth day almost half of the samples stored in any of the conditions had been affected. A slight over-ripening was also detected at this time in ICAPI-17-COL; the phenomenon was more marked in the samples stored in air or CA with 0% CO_2. These observations would appear to show that susceptibility to the spread of *Botritis cinerea* depends on the variety and not on the storage conditions (concentration of CO_2) provided; while over-ripening is probably slowed down by the presence of CO_2.

4. Conclusions

On the basis of the physiological and pathological changes noted, the fruits revealed a suitability for about 3 weeks storage in refrigerated CA, especially in the case of cv. ICAPI-30-COL.

Moreover, for ICAPI-17-COL the development during storage of some ripening parameters (firmness, TTA, pH and RD) seemed to be related to the concentration of CO_2 in the atmosphere, and showed a strong varietal influence. This correlation was not noted, however, for the weight decrease and spread of *Botritis cinerea*.

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The Use of Soya Bean, Sunflower and Lupin Seeds in the Preparation of Protein Bases for Nutritious Beverages

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Seed meal of soya bean, sunflower and lupin, and mixtures were examined for the preparation of protein bases suitable for nutritious beverages. Single step and successive extractions with aqueous calcium hydroxide solutions and water effect complete extraction of meal nitrogen. The calcium hydroxide extracted proteins of meal samples had an isoelectric pH of 5.0 at which 63–81% of the soluble protein was precipitated. Two successive extractions with calcium hydroxide and water had an isoelectric pH range of 4.4–4.6 and give maximum precipitation of 67% of the extracted protein. A detailed procedure is given for the preparation of creamy or light greyish coloured protein, readily soluble in water, with improved amino acid pattern.

Keywords: Soya bean; sunflower; lupin; nutritious beverage; protein isolates; amino acid pattern.

1. Introduction

In recent years active research and development programmes started worldwide to promote the production of nutritious beverages as well as other types of unconventional protein foods. Several carbonated and non-carbonated nutritious beverages have already found their way to the world market. Most of these use soya bean as a source of protein.

Processes used for the preparation of protein bases for nutritious beverages involve two major steps: (a) the extraction of protein from its source, and (b) the recovery of the solubilised protein. Sodium hydroxide is the most commonly used solvent for the extraction of proteins from vegetable sources by single step, successive or countercurrent extraction procedures.[1–3] However, the major problem involved with the use of sodium hydroxide is the dark colour of the extracts and the protein product resulting therefrom. Taha *et al.*,[4] prepared light coloured protein isolates suitable for nutritious beverages, by extracting seed proteins with aqueous calcium hydroxide solutions.

The present investigation was designed to utilise soya bean, sunflower and lupin seed protein for the preparation of a suitable protein base.

2. Experimental

2.1. Material

Soya bean (*Glycine max*), sunflower seed (*Helianthus annus*) and lupin seed (*Lupinus albus*) were locally grown varieties. Soya bean meal was prepared from the whole seed, sunflower seed meal from the dehulled seed, and lupin meal from the debittered-dehulled seed.[3] The ground seed samples were extracted with *n*-hexane and the meal samples were finely ground to pass through a 0.2 mm screen.

2.2. Analytical procedure

Moisture, lipids, crude fibre, ash and nitrogen-free extract were determined according to standard procedures.[5] A semimicrokjeldahl procedure was used for the determination of nitrogen,[6] protein

was calculated as N×6.25. Amino acid composition was determined on hydrolysates according to Moore *et al.*,[7] and Spackman *et al.*[8].

2.3. Protein extraction

(a) Single step extraction of defatted meals were carried out with 10 vol. of aqueous calcium hydroxide solutions of different concentrations. The mixture was vigorously stirred for 20 min at room temperature (28°C), followed by centrifuging at 6000×*g* for 125 min. The residue was discarded and the extract analysed for protein solubility based on actual recovery.

(b) Two successive extractions were carried out by re-extracting the residue with water. The residue from the second extraction was discarded, and the supernatant solution analysed for protein solubility. Solvent:meal ratios were taken on the basis of the original weight of meal sample.

2.4. Precipitation of proteins

The precipitation patterns were carried out on protein extracts of (a) soya bean, (b) soya bean–sunflower, (c) soya bean–sunflower–lupin, (d) mixtures, according to El-Nockrashy *et al.*[1]

2.5. Process for the preparation of protein bases

Samples a, b and c were used as the starting material for the preparation of protein bases for nutritious beverages. The process illustrated in Figure 1 has been developed to prepare the protein bases.

The following steps are involved in the preparation of the protein bases.

Protein extracts: finely ground meal samples (200 g a, b and c) were extracted first with 0.1 M calcium hydroxide (2 litres) then with water (2 litres). The resulting extracts X_I and X_{II} were mixed together and the combined extract adjusted to pH 11.5 with 3 M sodium hydroxide, then heated in a water bath at 70°C with gentle stirring for 30 min.

Protein curd: the protein alkaline solution was adjusted to pH 4.6 using 3 M phosphoric acid solution. The protein curd was centrifuged at 3000×*g* and the supernatant was discarded. The curd was washed with water once at a solvent to meal ratio of 15:1 and a second time at a ratio of 1:1 (by vol.), by stirring gently for 10 min before centrifuging. The wash was discarded.

Protein neutralisation: the washed protein was mixed with 500 ml water and adjusted to pH 6.8 with 3 M sodium hydroxide solution. The neutral protein was heated by steaming in an autoclave for 5 min. The solubilised protein was lyophilised to give a transparent needle-like product.

3. Results and discussion

Calcium hydroxide solutions proved to be efficient for the extraction of proteins from soya bean, sunflower and lupin seeds and yield lighter coloured protein isolates than sodium hydroxide protein extracts.[4]

The highest solubility of protein from soya bean, sunflower and lupin seed meals (99, 64, and 84% respectively) was achieved by two successive extractions, first with calcium hydroxide solution (0.03, 0.10, and 0.20 M respectively) and then with water, both at a solvent:meal ratio of 10:1.

As the aim of the investigation was to use a mixture of the three seed meals that would offer better amino acid patterns, the design was planned to include: (a) a study of the amino acid contents of the three seed meals, (b) choices for ratio formulations of seed meals, (c) solubility of mixed meals, and (d) protein precipitation of the mixed meals.

3.1. Amino acid pattern of seed meal

Table 1 gives the amino acid contents of soya bean seed meal sunflower seed meal and lupin seed meal, and their percentage of FAO requirements.

The data reveal that soya bean is rich in lysine, isoleucine and tyrosine+phenylalanine. Sunflower is rich in valine, sulphur amino acids, isoleucine and tyrosine+phenylalanine, while lupin is rich in isoleucine and tyrosine+phenylalanine.

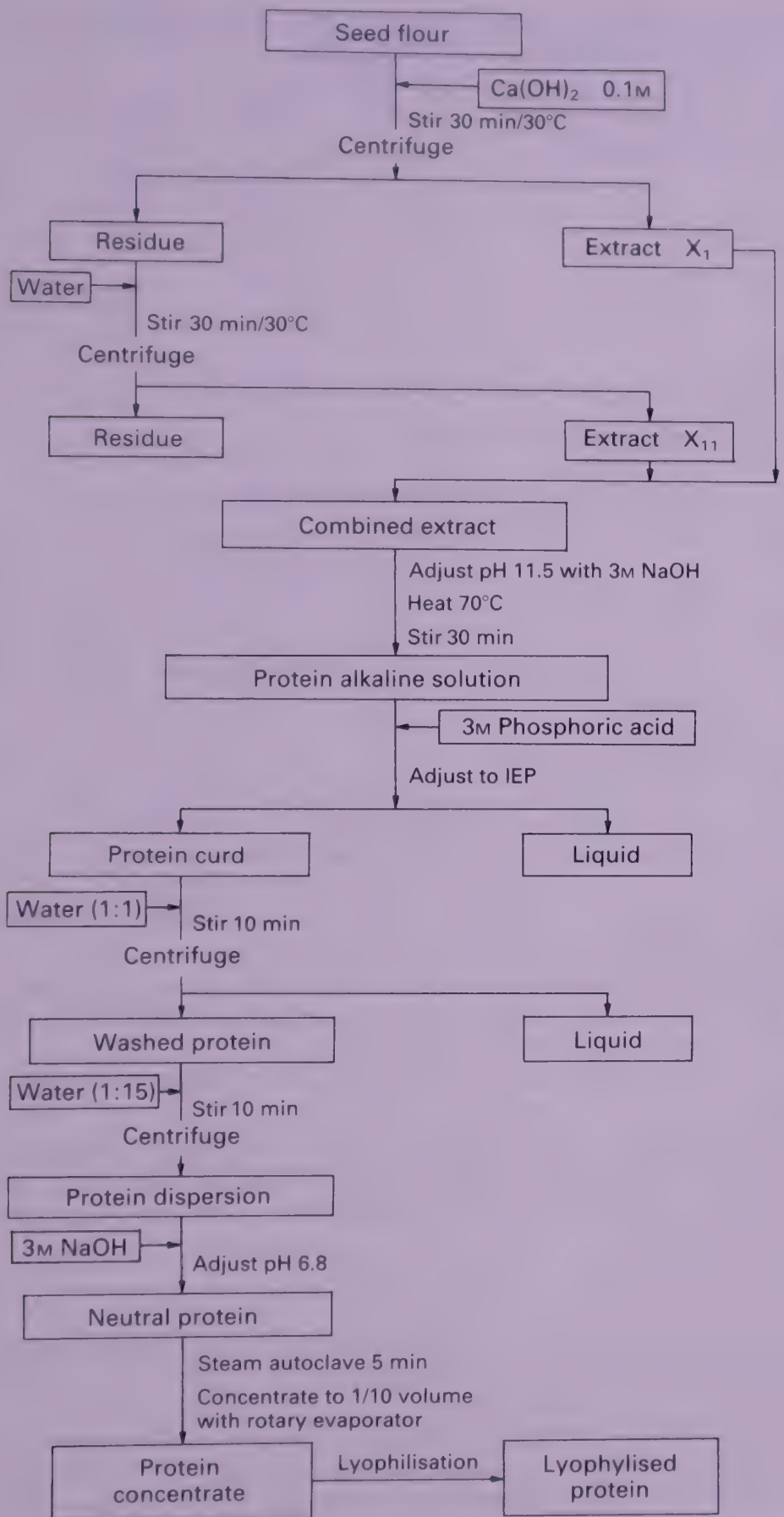

Figure 1. Process for the preparation of the protein base.

Lysine is a limiting amino acid in sunflower and lupin (71 and 78% of the FAO requirement) while soya bean is a rich source (105% of the requirement). Sulphur amino acids are limiting in soya bean and lupin (77 and 57% of the requirement), while sunflower is rich in sulphur amino acids (111% of the requirement). Valine is a limiting amino acid in soya bean and lupin (84 and 78% of the

Table 1. Amino acid content of soya bean, sunflower and lupin seed meals and their percentage of the FAO requirement[9]

Amino acid	Soya bean		Sunflower		Lupin		FAO requirement
	g 16 g^{-1} N[a]	FAO (%)[b]	g 16 g^{-1} N	FAO (%)	g 16 g^{-1} N	FAO (%)	
Lysine	5.8	105	3.9	71	4.3	78	5.5
Threonine	4.0	100	4.0	100	3.8	95	4.0
Valine	4.2	84	5.2	104	3.9	78	5.0
Methionine+ cysteine	0.8 1.9	77	2.6 1.3	111	0.4 1.6	57	3.5
Isoleucione	4.7	118	5.2	130	4.6	115	4.0
Leucine	6.6	94	7.0	100	7.0	100	7.0
Tyrosine+ phenylalanine	4.1 5.7	163	2.6 5.4	133	5.7 3.5	153	6.0

[a] Amino acids calculated as g 16 g^{-1} N.
[b] Amino acids calculated as percentage of FAO requirements.

Table 2. Calculated values for the amino acid contents of three samples A, B and C[a,b]

Amino acid	A	B	C
Lysine	105	98	99
Threonine	100	100	100
Valine	84	88	85
Methionine+cysteine	77	84	78
Isoleucine	118	120	119
Leucine	94	95	95
Tyrosine+phenylalanine	163	157	159

[a] A, Soya bean meal; B, soya bean meal–sunflower meal (8:2); C, soya bean meal–sunflower meal–lupin meal (8:1:1).
[b] Values are calculated as the percentage of FAO requirements.

requirement), while in sunflower it constitutes 104% of the requirement. All other essential amino acids are found in adequate quantities in the three meal samples.

Based on these findings, three seed meal samples were considered as starting materials for protein extraction: sample A—soya bean, sample B—soya bean and sunflower (8:2, by wt) and sample C—soya bean, sunflower and lupin (1:1:1 by wt).

Table 2 represents the calculated values for the amino acid contents of the three samples as the percentage of the FAO reference pattern.

Mixtures B and C have the advantages of (a) increased lysine content compared with sunflower and lupin, (b) increased sulphur amino acid content compared with soya bean and lupin, and (c) increased valine content compared with soya bean and lupin. However, they are still limiting in sulphur amino acids and valine.

3.2. Solubility of mixed protein

Table 3 gives the protein solubilities of seed meal samples in calcium hydroxide solutions of concentration range 0.03–0.2M, at a solvent:meal ratio of 10:1. Protein solubilities of 95% or more were obtained with 0.1 M calcium hydroxide.

Two successive extractions of samples A, B and C first with 0.1 M calcium hydroxide then water, both at a solvent:meal ratio of 10:1, result in complete extraction of the meal nitrogen (Table 3). Values higher than 100% solubility means that the conversion factor of the protein should be less than 6.25.[10]

3.3. Precipitation of mixed protein extracts

Figure 2 represents precipitation curves of protein from calcium hydroxide extracts of samples A, B and C. Figure 3, illustrates the precipitation curves of protein from combined calcium hydroxide water extracts of the three samples.

Table 3. Solubility of samples A, B and C in calcium hydroxide and calcium hydroxide/water by single step and two successive extractions[a]

Sample	$Ca(OH)_2$ solutions (M)	pH	Solubility (%)
Single step extraction			
A	0.03	9.0	88.3
	0.05	9.8	92.6
	0.10	10.6	97.9
	0.20	11.2	96.1
B	0.03	9.6	85.0
	0.05	10.9	90.5
	0.10	11.7	96.7
	0.20	12.0	94.7
C	0.03	9.6	83.0
	0.05	10.9	91.6
	0.10	11.7	94.6
	0.20	12.0	93.1
Two successive extractions			
A	*First* 0.1 M $Ca(OH)_2$	9.1	93.9
	Second water	8.8	15.6
	Total		109.5
B	*First* 0.1 M $Ca(OH)_2$	11.6	94.5
	Second water	11.6	8.4
	Total		102.9
C	*First* 0.1 M $Ca(OH)_2$	11.6	92.6
	Second water	11.4	6.7
	Total		99.3

[a] A, Soya bean meal; B, soya bean–sunflower meal (8:2, by wt); C, soya bean–sunflower–lupin meal (8:1:1, by wt). Solvent:meal ratio=10:1.

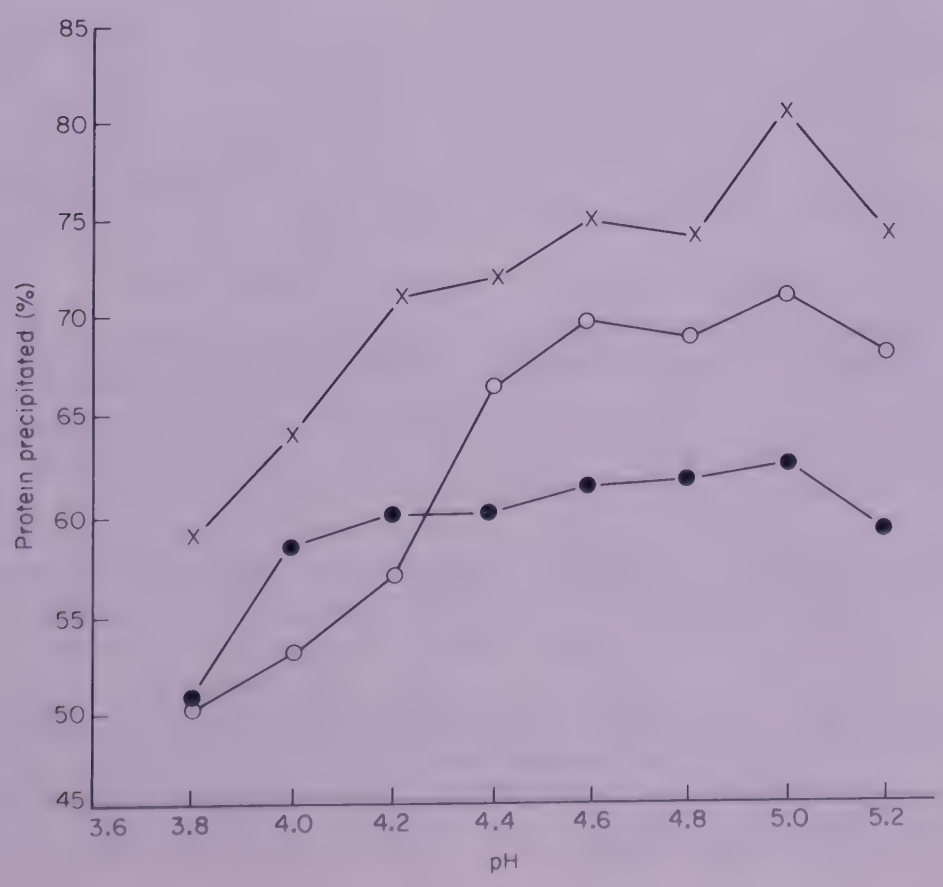

Figure 2. Isoelectric precipitation curve of calcium hydroxide extracts: (○), soya bean; (●), soya bean–sunflower; (×), soya bean–sunflower–lupin.

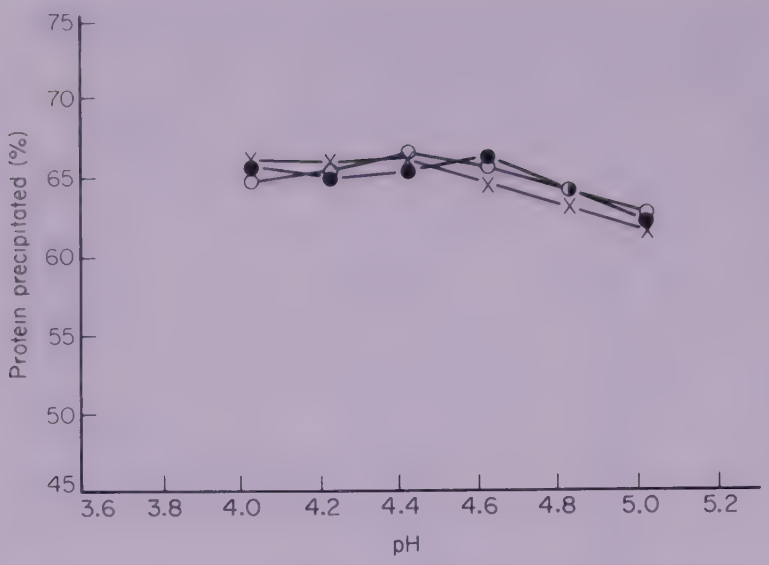

Figure 3. Isoelectric precipitation curve of the combined calcium hydroxide/water extracts (two successive extractions): (○), soya bean; (●), soya bean–sunflower; (×), soya bean–sunflower–lupin.

Proteins extracted from the three samples with calcium hydroxide give precipitation curves with an isoelectric point at pH 5.0 where 63–81% of the soluble protein (60–70% of the meal protein) is precipitated. Two successive extractions first with calcium hydroxide, then with water result in similar protein precipitation patterns with isoelectric points between pH 4.4 and 4.6, and with maximum precipitation yields of about 67% of the protein from the extracts. Although this value is lower than that obtained with single step extraction the amount of protein precipitated is higher because the protein content of the extracts resulting from two successive extractions is 7–16% higher than the calcium hydroxide extracts of a single step.

3.4. Protein bases prepared for beverages

Two successive extractions of samples A, B and C first with 0.1 M calcium hydroxide, then with water result in a combined extract that contains 98 to 100% of the original meal nitrogen.

Based on a recommendation made by Magnino and Frederiksen,[11] in their study for preparation of soluble protein products the pH of the combined extracts was adjusted with 3 M sodium hydroxide to a value of 11.5 followed by heating at 70°C for 30 min (Figure 1). It was found that this treatment results in a final protein base that is completely soluble in water. Addition of sodium hydroxide at this stage does not affect the colour of the solution or the final product.

The useof 3 M phosphoric acid in the precipitation of proteins curd at the IEP results in improvement of the colour of the final product, over the use of HCl.

Washing of the protein curd with water improves the colour of the final product and results in less than 5% loss in the final yield of protein base.

3.5. Chemical and amino acid composition of protein bases

Table 4 gives the chemical composition of the meal samples A, B and C, and the protein bases prepared therefrom, following the procedure given in Figure 1.

Table 4. Analysis of meal samples and protein base prepared therefrom[a,b]

	A		B		C	
	M	P	M	P	M	P
Protein (%)	46.2	81.3	46.7	83.8	47.61	85.3
Oil (%)	0.1	5.1	0.1	5.1	0.1	2.7
Ash (%)	7.2	4.9	7.6	4.5	6.8	6.3
Crude fibre (%)	5.5	0.4	6.0	0.2	6.8	0.3
NFE (%)	41.0	8.3	39.6	6.4	38.7	5.4

[a] Values are given on moisture free basis.

[b] A, Soya bean; B, soya bean–sunflower; C, soya bean–sunflower–lupin; M, meal; P, protein product prepared therefrom

NFE, nitrogen free extract.

The three protein preparations are transparent, needle-like and cream in colour, or with a light greyish tint, and readily soluble in water. The data from Table 4 reveal that the three protein bases contain from 81 to 85% protein which is double or triple the amount present in the original meal samples. Oil residues are in the range of 3–5%, the nitrogen free extract, including sugars, 5–8% and the ash content 4.5–6%. Crude fibre constitutes less than 0.5%.

Table 5 gives the amino acid analyses of the three protein preparations and Table 6 the calculated amino acid contents relative to FAO requirements for human adults. The values are given for meal samples A, B and C, and the protein products prepared therefrom.

Table 5. Amino acid composition of the three protein preparations[a, b]

Amino acid	A	B	C	FAO requirement[a]
Lysine	6.1	5.0	4.3	5.5
Threonine	4.0	3.9	4.1	4.0
Valine	4.2	4.9	5.6	5.0
Methionine	0.7	1.3	1.1	3.5
Cysteine	0.9	0.9	0.9	
Isoleucine	4.4	4.1	4.9	4.0
Leucine	8.9	10.3	9.8	7.0
Tyrosine	3.4	3.6	3.5	6.0
Phenylalanine	5.7	7.1	5.7	

[a] Values are given as g 16 g^{-1} N.

[b] A, Soya bean product; B, soya bean–sunflower product; C, soya bean–sunflower–lupin product.

Table 6. Amino acid composition of meal samples and protein products prepared therefrom[a, b]

	A		B		C	
Amino acid	M	P	M	P	M	P
Lysine	105	111	98	100	99	78
Threonine	100	100	100	98	100	103
Valine	84	85	88	98	85	112
Methionine+cysteine	77	45	84	64	78	58
Isoleucine	118	110	120	102	119	122
Leucine	94	124	95	147	95	140
Tyrosine+phenylalanine	163	151	157	178	159	153

[a] Values are given as percentage of FAO requirements.

[b] A, Soya bean; B, soya bean–sunflower (8:2, by wt); C, soya bean–sunflower–lupin (8:1:1, by wt); M, meal; P, protein product prepared therefrom.

The results reveal that (a) protein base prepared from soya bean has higher lysine and leucine contents than the meal; while the sulphur amino acids content decreases by about 41%. Therefore, the protein base prepared from soya bean meal is first limiting in sulphur amino acids. (b) Protein base prepared from soya bean:sunflower (8:2) has higher valine, leucine and tyrosine+ phenylalanine than the meal mixture. The protein base constitues better composition of amino acids than the protein base prepared from soya bean meal alone. Though the soya bean–sunflower protein base contains higher sulphur amino acid than the base prepared from soya bean alone, the mixed base is still limiting in sulphur amino acids. (c) Mixed meal of soya bean–sunflower–lupin (8:1:1) produces a protein base that is limiting in both sulphur amino acids and lysine. Other essential amino acids are present in adequate amounts.

It is therefore concluded that the use of mixed soya bean–sunflower seed meal (8/2, by wt) in the production of a protein base for a nutritious beverage offer the following advantages: Complete solubility of mixed meal nitrogen with two successive extractions first with 0.1 M calcium hydroxide, followed by water both at a solvent:meal ratio of 10:1; overall recovery of about 68% of the mixed

meal protein as a protein base; the protein base produced contains about 84% protein; although the protein base is still limiting in sulphur amino acid (64% of the FAO requirement) the value is considerably higher than the base prepared from soya bean alone (45% of requirement).

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Pepino (*Solanum muricatum*): Chemical Composition of Ripe Fruit

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Ripe pepino fruit (*Solanum muricatum* L. cv. 'El Camino') were analysed for water, sugars, non-volatile organic acids, amino acids, starch, vitamin C, minerals and cell wall composition. The sugars consisted of sucrose, fructose and glucose with sucrose accounting for 50% of the total. Citric acid made up more than 90% of the non-volatile organic acids. Malic acid and traces of quinic acid were also present. Aspartic acid accounted for 70% of the total free amino acids. Cellulose, pectin and hemicellulose represented 71, 17 and 11% respectively of the cell wall polysaccharides. The vitamin C level was higher than normally found in most fruits, including citrus. A limited study suggested that the major change during maturation was a continuous increase in the levels of sucrose, citric acid and the dicarboxylic amino acids.

Keywords: Pepino; chemical composition; sugars; organic acids; amino acids; cell wall polysaccharides.

1. Introduction

The pepino (*Solanum muricatum*) is a small herbaceous perennial member of the Solanaceae. It is native to the Northern Andes region of South America where it is grown at altitudes up to 2700 metres above sea level. In New Zealand it is grown as an annual and propagated vegetatively. Pepinoes vary from round to elongate, are of 5 to 20 cm in length and are generally yellow with purple stripes. They are sweet and juicy and may be eaten singly or as a dessert fruit in a similar manner to rock melons.

The pepino has been developed as a commercial crop in Peru and Chile and recently in New Zealand and Australia. In New Zealand, a small export market to Japan and the USA has been established but the future growth of this fragile industry will depend on establishing criteria for consistent quality of fruit. Improvement of quality in general requires more basic information[1] on the nutrition, physiology and chemistry of the pepino. The results of a study on the chemical composition of fruit of *Solanum muricatum* L. cv. 'El Camino' are presented in this paper.

2. Experimental

2.1. Samples

Twenty pepinoes of prime market quality were taken from a commercial orchard near Auckland. The fruit were harvested randomly from different positions on 20 plants. They were separated into four samples of five fruit and the compositional analysis done on each of the four samples.

2.2. Extraction and determination of sugars, organic acids and amino acids

The peeled fruit was divided into edible pericarp, which accounted for 90% of the fruit weight, and the core tissue. The latter consisted of the septum, placental tissue, seeds and small amounts of pericarp tissue and represented 6–8% of the fruit weight. The sliced pericarp (~600 g per sample)

was immersed in liquid nitrogen in a Waring blender; methanol:formic acid (400 ml, 9:1 by vol) was added and the mixture homogenised for 30 s. The homogenate was transferred to a stainless steel beaker and thoroughly homogenised in a Kinematica Polytron for 2 min to produce a finely dispersed suspension. A sub-sample (100 g from ~750 g homogenate) was transferred to a flask containing methanol:chloroform (230 ml, 15:8 by vol), left overnight at −20°C and the sugars, non-volatile organic acids and amino acids were extracted, purified and determined quantitatively by procedures described elsewhere.[2–4]

The smaller core tissue samples (~50 g per sample) were immersed in liquid nitrogen; methanol:chloroform:formic acid:water (MCWF, 50 ml, 12:5:1:1 by vol) was added and the tissue dispersed by a Polytron for 2 min. The samples were left overnight at −20°C and the sugars, organic acids and amino acids extracted and determined as for the pericarp.

2.3. Determination of starch and cell wall polysaccharides

The residues of the extracted tissues contained cell wall material (CWM) and small amounts of starch. The latter was solubilised in 90% dimethyl sulphoxide and recovered by dialysis and lyophilisation. The starch content was determined by enzymic hydrolysis with amyloglucosidase.[5]

The de-starched CWM of the four pericarp samples was combined (~1 g) and the pectic and hemicellulosic polysaccharides extracted sequentially with 0.05 M CDTA (1,2-cyclohexanediaminotetraacetic acid), 0.05 M Na_2CO_3 at 4°C and 4 M KOH.[6] The polysaccharides were recovered by dialysis and lyophilisation. Their sugar composition was determined by g.l.c. as the alditol acetates following Saeman hydrolysis of the polysaccharides.[7]

2.4. General analysis

Protein was determined by the Kjeldahl method (%N×6.25) applied to a lyophilised sample of the pericarp homogenate which had been dialysed to remove free amino acids and inorganic nitrogen. The lipid and pigment fraction was assumed to be equivalent to the total dissolved solids in the chloroform phase of the MCWF extracts and it therefore may be overestimated. Elemental analysis was carried out on lyophilised samples of the tissue homogenates by an analytical services laboratory using standard procedures. The vitamin C content was measured on each of six freshly harvested fruit by the 2,6-dichlorophenolindophenol method.[8]

3. Results and discussion

3.1. Sugar composition

The dry matter of the pericarp tissue of pepino varied between 6.8–8.2% (Table 1) and of this ~75% occurred as sucrose, fructose and glucose. The sugar content of pepinoes on a fresh weight basis is therefore of a similar order to that found in currants, melons and strawberries.[9] Sucrose accounted for slightly more than 50% of the total sugar with fructose and glucose contributing 28% and 18% respectively (Table 2). The core tissue contained similar amounts of fructose and glucose as the pericarp but nearly twice the concentration of sucrose. Inositol was detected in very small amounts (<0.01%) in both tissue zones. Detectable levels of other oligosaccharides (e.g. raffinose) were not found despite attempts to isolate them using preparative paper chromatography. Average brix values for each of the four fruit samples were between 7.8–9.6. However the total sugar as estimated by g.l.c. was between 4.9 and 6.4 g 100 g^{-1} of tissue, indicating that soluble solids can be used only as a rough guide to sugar levels in pepinoes.

3.2. Non-volatile organic acid composition

Citric acid accounted for 91% of the total non-volatile organic acids found in the fruit (Table 2). Other acids detected by g.l.c. were malic and quinic acid. The identity of all acids was confirmed by t.l.c. Oxalic acid was not found despite the use of extraction procedures specifically designed to isolate this acid.[10]

Table 1. Concentration range of constituents found in pericarp tissue of ripe pepino fruit

Constituent	[a] Amount (values per 100 g fresh tissue)
Dry matter (g)	6.8–8.2
Protein (g)	0.10–0.13
Lipid and pigment (mg)	24.6–44.4
Soluble sugars (g)	4.9–6.4
Starch (mg)	20.0–90.0
Cellulose (mg)	154–220
Hemicellulose (mg)	40.1–53.6
Pectin (mg)	26.7–34.5
Vitamin C (mg)	48.0–68.8
Non-volatile organic acids (mg)	119–153
Free amino acids (mg)	52–70
N (mg)	23–30
P (mg)	10.7–12.3
K (mg)	115–123
S (mg)	3.0–4.0
Ca (mg)	2.3–3.0
Mg (mg)	5.3–6.1
Na (mg)	0.76–2.30
Fe (mg)	0.20–0.31
Mn (mg)	0.06–0.07
Zn (mg)	0.02–0.05
Cu (mg)	0.02–0.03
B (mg)	0.03–0.05

[a] Represents the concentration spread found in the four samples of fruit.

Table 2. Sugar and organic acid content of two tissue zones of ripe pepino fruit

Constituent	[a] Amount (values per 100 g fresh tissue)	
	Pericarp	Core
Fructose (g)	1.4±0.05	1.3±0.1
Glucose (g)	0.9±0.02	0.8±0.06
Sucrose (g)	2.6±0.32	4.9±0.61
Inositol (g)	<0.01	<0.01
Malic acid (mg)	10.1±0.23	14.8±0.9
Citric acid (mg)	124.2±7.5	180.0±8.3
Quinic acid (mg)	1.4±0.1	1.5±0.1

[a] Mean value ±s.e. of four samples of fruit.

3.3. Amino acid composition

The free amino acids were separated by two-dimensional t.l.e. and t.l.c. and estimated spectrophotometrically.[3] Twenty-five ninhydrin positive compounds were detected of which eighteen were identified as commonly occurring amino acids (Table 3). Pipecolic acid was also identified. The other six compounds were not identified but the ninhydrin reaction of one of them suggested the presence of an indole derivative. None was a major component. Aspartic and glutamic acids comprised 90% of the total free amino acids in ripe pepinoes with aspartic acid making the larger contribution. Qualitatively there were no differences between the two tissue zones, but the concentration of aspartic acid in the core tissue was twice that of the pericarp. The nitrogen status of the plant and its inorganic nitrogen form are known to influence the amounts and proportions of the free amino acids found in fruit tissues.[9] The total N for the pepino was low (30 mg 100 g $^{-1}$) when compared to published values for a range of fruits (120–300 mg 100 g $^{-1}$),[9] suggesting perhaps that the

Table 3. Free amino acid constituents of two tissue zones of ripe pepino fruit

Amino acid	[a]Amount (values mg per 100 g fresh wt)	
	Pericarp	Core
Aspartic acid	33.2±4.9	75.3±9.9
Glutamic acid	10.3±2.5	12.9±1.1
Glutamine	0.7±0.18	1.8±0.3
Histidine	0.6±0.25	4.0±1.5
Proline	1.4±0.20	<0.1
Lysine	0.2±0.08	0.7±0.2
Alanine	0.3±0.08	0.3±0.04
Serine	0.4±0.08	0.5±0.1
γ-Aminobutyric acid	0.2±0.07	0.4±0.20
Threonine	0.2±0.07	0.1±0.01
Glycine	<0.01	<0.01
Valine	<0.01	<0.01
Leucine	<0.01	<0.01
Isoleucine	<0.01	<0.01
Phenylalanine	<0.01	<0.01
Methionine	<0.01	<0.01
Asparagine	<0.01	<0.01
Cysteine	<0.01	<0.01
Pipecolic acid	n.d.	n.d.

[a]Mean value ±s.e. of four samples of fruit.
n.d. Not determined.

plants may have been nitrogen deficient. However, a leaf analysis showed that the plants contained levels of nitrogen that were adequate for healthy growth. The plants were grown in soil containing nitrate as the major form of inorganic nitrogen.

3.4. Cell wall composition and starch content

The sugar composition of the CWM of the pericarp and core tissues is shown in Table 4. Glucose and uronic acid were the major components of the pericarp polysaccharides with mannose and xylose the main non-cellulosic neutral sugars. The core tissue showed a decreased proportion of glucose and increased amounts of uronic acid and mannose. Fractionation of pericarp CWM with solutions of CDTA, Na_2CO_3 and KOH yielded fractions of ionically bound pectin, covalently bound pectin and hemicellulose, respectively.[6] The two pectic fractions accounted for 12% of the CWM. Following partial acid hydrolysis of the pectin the disaccharide acid rhamnogalacturonic acid was tentatively identified. This finding, together with the sugar composition of the pectin fractions (Table 5),

Table 4. Sugar composition of cell wall material of ripe pepino fruit

Sugar residue	Pericarp tissue		Core tissue	
	Content[a] μg mg^{-1} CWM[b]	Percentage of the total sugar content	Content μg mg^{-1} CWM	Percentage of the total sugar content
Rhamnose	9.3±0.3	1.3	7.3±0.5	1.6
Arabinose	38.8±1.6	5.4	28.3±1.5	6.5
Xylose	44.9±1.4	6.2	32.0±1.0	7.3
Mannose	45.8±2.2	6.4	61.4±5.1	14.0
Galactose	35.2±2.3	4.9	33.1±3.0	7.5
Glucose	421.0±9.8	59.2	149.3±10.5	34.1
Uronic acid	121.0±6.6	16.9	126.5±8.5	28.9

[a]Mean value ±s.e. of four samples of fruit.
[b]The CWM represents 0.34% of the fresh weight and 4.4% of the dry weight of the pericarp tissue. The respective values for the core tissue are 0.68% and 6.6%.

Table 5. Sugar composition of pepino pericarp cell wall fractions

Fraction	CWM %	[a]Sugar composition (μg mg^{-1} CWM)						
		Rha	Ara	Xyl	Man	Gal	Glc	Uronic acid
CDTA	7.0	17	150	5.3	–	49	26	493
Na_2CO_3	5.0	18	154	6.2	–	57	15	400
KOH	19.9	–	45	205	65	69	176	80
Residue	68.1	3	15	12	33	18	416	34

[a] Mean value of duplicate determinations.
Rha=rhamnose, Ara=arabinose, Xyl=xylose, Gal=galactose, Glc=glucose.

suggested the presence of the well established rhamnogalacturonan backbone[11] as the main feature of the pectic polysaccharides. The hemicellulose fraction (20% CWM) contained xylose and glucose as the main sugars, indicating that the major hemicellulose of pepino cell walls may be a xyloglucan of the type commonly reported to occur in the walls of many dicotyledenous plants.[11]

The starch content of ripe pepinoes was low but appeared to be concentrated in the core tissue region of the fruit (20–90 mg 100 g^{-1} pericarp, 170–770 mg 100 g^{-1} core).

3.5. Compounds of nutritional significance

The pepino contained vitamin C at higher levels (Table 1) than normally found in most fruits (including citrus). The vitamin C content of pepino is therefore of nutritional significance in relation to the recommended daily allowance.[12] However the concentrations of protein, dietary fibre and minerals (except possibly for potassium at 120 mg 100 g^{-1}, Table 1) are not of nutritional significance.

3.6. Changes in chemical composition during maturation

The pericarp of pepinoes harvested at three stages of maturity was extracted with MCFW and the sugar, non-volatile organic acid and dicarboxylic amino acid levels compared (Table 6). A marked increase in the sucrose content of the fruit as it matured was reflected in a decrease in the ratio of reducing sugar to sucrose in stages 1, 2 and 3. (50:1, 3:1, 1:1 respectively). The total non-volatile organic acids increased slightly as the fruit matured but the biggest change occurred in the relative amounts of malic and citric acid. In stage 1 fruit the malic and citric acid concentrations were similar (30–40 mg 100 g^{-1}) but at stage 3 the malic acid was lower (10 mg 100 g^{-1}) while the citric acid content had increased by 300% (124 mg 100 g^{-1}). Quinic acid, which represented only 1% of the total acid in stage 1 fruit, accounted for 21% of the total acid in stage 3 fruit, accounted for 21% of the total acid in stage 1 fruit. The dicarboxylic amino

Table 6. Changes in sugar, organic acid and dicarboxylic amino acid constituents of pepino fruit harvested at three stages of maturity

Constituent	[a]Maturity stage (values mg 100 g^{-1} tissue)		
	1	2	3
Fructose	1890	1550	1440
Glucose	1200	990	930
Sucrose	63	840	2620
Inositol	100	10	10
Malic acid	32	12	10
Citric acid	40	80	124
Quinic acid	20	4	1
Aspartic acid	3	3	33
Glutamic acid	5	3	10

[a] 1, Represents fruit estimated to be 6 weeks from the maturity stage of 3 (Brix reading 5.0); 2, represents fruit estimated to be 2 weeks from the maturity stage of 3 (Brix reading 6.2); 3, represents ripe fruit (Brix reading 9.3).

acids showed a five-fold increase in concentration as the fruit matured but, whereas aspartic acid was the predominant amino acid in ripe fruit, glutamic acid was present in slightly higher amounts in the younger fruit.

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Peptic Hydrolysis of Gluten, Glutenin and Gliadin from Wheat Grain: Kinetics and Characterisation of Peptides

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The kinetics of the hydrolysis of gluten, glutenin and gliadin by pepsin was studied by following the reaction of the hydrolysate with ninhydrin and by SDS-gel electrophoresis. Glutenin was more rapidly hydrolysed than native molecules of gliadin and produced components of different size. The first stage of hydrolysis of gliadin resulted in the formation of fragments mainly in the range 25 000 and 10 000 daltons whereas polypeptides generated from glutenin had subunits of molecular weight (MW) in the range 65 000, 30 000 and 10 000. Further hydrolysis resulted in the formation of subunits of MW around 24 000 and 14 000 for glutenin and gliadin respectively. The final stage of hydrolysis produced a quantity of peptides of MW under 10 000 and a small quantity of free amino acids. No obvious differences in the molecular weights of the polypeptides released were found when the enzyme to gliadin ratio was varied. Components obtained from glutenin and gliadin were further characterised after gel filtration on Sephadex G50. Large polypeptides derived from both gliadin and glutenin contained large amounts of proline and glutamate. Surface hydrophobicity of derived components were different, and inferior to, those of the reduced glutenin and native gliadin. Reduction of disulphide bonds of digested polypeptides of glutenin showed the existence of peptides branched by interchain disulphide bonds. Peptic cleavage sites were probably located differently in glutenin and gliadin molecules.

Keywords: Hydrolysis; pepsin; gluten; glutenin; gliadin; peptides.

1. Introduction

Enzymic hydrolysis of proteins seems to be a valuable method for the preparation of polypeptides and peptides with new functional and biological properties.[1, 2] Hydrolysates as well as dietary protein have been shown to be of interest as a source of N in chemically-defined elemental diets.[3]

Enzymic hydrolysis of wheat protein has been used to investigate flour quality in relation to its physical properties,[4] both in order to characterise free amino acids contents for nutritional experiments,[5–8] and to gain a better knowledge of the structure of gliadin and glutenin[9–12] or at least to compare partial amino acid sequences of wheat proteins with those of other cereals.[13] Other studies concerning enzymic digestions of gluten concentrated on isolating active peptides which may be toxic in coeliac disease.[14–16] Because of reports linking wheat gluten digestion with mental disorders[17,18] purified peptides from peptic digests of wheat gluten were tested for opioid-like activity.[19, 20] Few reports are concerned with the precise description of components produced by enzymic hydrolysis of wheat proteins and modification of these proteins in order to obtain new functional properties.

Among proteinases, porcine pepsin is known to have a broad side chain specificity[21] and to produce large polypeptides from wheat proteins.[12, 22] In order to gain a better understanding of the different components resulting from the peptic digestion of wheat proteins, their two main fractions, glutenin and gliadin, were digested by pepsin under controlled conditions and the resulting components were analysed by different techniques. The results obtained would give some information on

how to prepare polypeptides and peptides possessing various functional characteristics according to their molecular weights, amino acid composition and hydrophobicity.

2. Experimental

2.1. Preparation of wheat protein fractions

Partially defatted gluten of common wheat *Triticum aestivum* (cv. Capitole) was prepared according to Popineau.[23] Crude gliadin was extracted from gluten by solubilisation in a water–dioxane solvent (40:60 by vol). Crude gliadin and glutenin (the undissolved material) were directly freeze-dried.

The purified gliadin fraction was prepared by large-scale gel filtration on Ultrogel Aca 34 (column diam. 10 cm, height 77 cm, sample size 5 g) equilibrated with 0.1 M acetic acid, 2 M urea, 0.05 M KCl, pH 3.3 at a flow rate of 300 ml h^{-1} (Figure 1). Excluded peak 1 accounted for 38.8% of the total protein representing soluble glutenins. Peak 2 consisted of gliadin and accounted for 47.1% of recovered proteins. The fractions corresponding to this peak were pooled and constituted purified gliadin. Peak 3 containing albumins and globulins and non-protein components was discarded. The entire gliadin fraction was dialysed against distilled water and freeze-dried.

The protein content (N×5.7) of the defatted gluten, the purified gliadin and the crude glutenin were 82.3, 93.1 and 70.1% of dry matter respectively.

For peptic digestion, protein fractions (750 mg) were dispersed in 21 ml 0.1 M acetic acid adjusted to pH 2 with 1 M HCl. Crystalline porcine pepsin (2000 FIP g^{-1}: Merck) was added in the enzyme/substrate ratios: 1/20, 1/33, 1/50, 1/100 or 1/1000 and the enzymic digestion carried out at 20°C. Samples (0.5 ml) were taken from the reaction batch at controlled times and mixed with 25 ml 0.05 M phosphate buffer pH 7.2 for the ninhydrin reaction of 1 ml tris buffer pH 7.5 (0.01 M tris containing 1.6% SDS, 1% 2-mercapto ethanol) for electrophoresis analysis. For analysis by gel filtration, the reaction was stopped by adjusting the hydrolysate to pH 7.2 with concentrated ammonia.

2.2. Chromatographic techniques

Gel filtration experiments were performed on a Sephadex G50 column (85×2.5 cm) equilibrated with 0.05 M ammonium acetate pH 7.2 Hydrolysate sample (10 ml) was centrifuged at pH 7.2 to remove undisolved material before it was applied to the column. Column flow rate was 25 ml h^{-1}. Fractions were detected simultaneously at 280 nm and 220 nm, pooled, concentrated *in vacuo* and freeze-dried.

Reversed phase high-performance liquid chromatography (RP-h.p.l.c.) was performed using a Kontron h.p.l.c. 600 system monitored by a series 200 programmer. Hydrolysates were fractionated on a Spherisorb S5 ODS C18 (12.5 cm×4.9 mm i.d.) supplied by Kontron. An acetonitrile–water gradient (buffer A: 0.05% (by vol) trifluoroacetic acid (TFA); buffer B: 0.05% (by vol) TFA, 75%

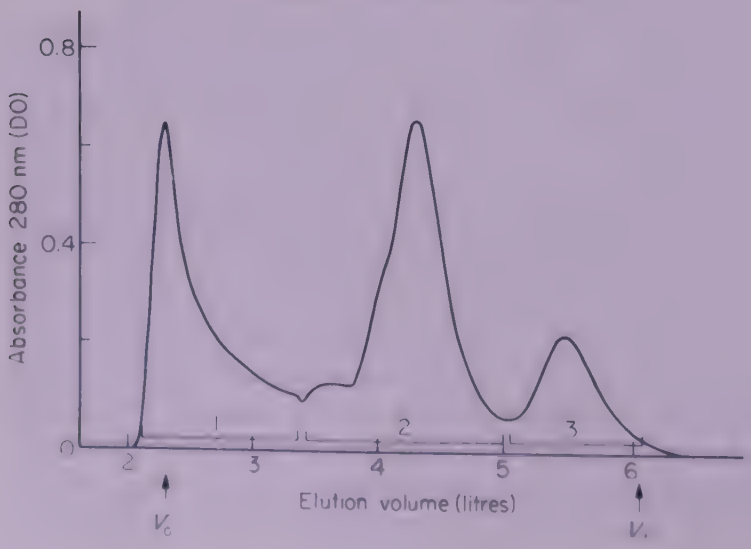

Figure 1. Fractionation of dioxane–water soluble proteins of gluten by gel filtration on Ultrogel ACA 34. The column was eluted with 0.1 M acetic acid, 2 M urea, 0.05 M KCl, pH 3.3.

(by vol) acetonitrile; pH 2.5) was used for the elution of peptides from the RP column. Solvent B concentration was increased from 0 to 100% in 60 min (1.25% B min^{-1}) at a flow rate of 1 ml min^{-1}. Samples were dissolved in buffer A (concentration 5 mg ml^{-1}) except for the native glutenin which was reduced by 5% (by vol) 2-mercaptoethanol in 8 M urea, tris 0.05 M buffer, pH 7.6, before being loaded on to the column. Proteins were detected at 214 nm with a Kontron Uvikon 730 LC detector using 0.5 absorbance units full scale.

2.3. Analytical methods

For the ninhydrin reaction, 1 ml of hydrolysate was mixed with 1 ml of ninhydrin reagent in a reaction bath at 100°C for 30 min. After cooling and centrifuging, 10 ml ethanol–water solution (50:50, by vol) was added. Absorbance was recorded at 570 nm. Leucine was used as a standard and the results expressed as leucine equivalents.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Tome *et al.*[24] but with a polyacrylamide gradient of 13–26% obtained by two solutions containing 13 g acrylamide plus 345 mg of NN′ methylene bisacrylamide and 26 g acrylamide plus 690 mg of NN′ methylene bisacrylamide each in 100 ml of 0.5 M tris buffer pH 6.8 respectively.

Densitometric patterns were recorded on a Vernon recording densitometer.

Amino acid analyses were made on a Kontron Liquimat III amino acid analyser after sample hydrolysis (1 mg ml^{-1}) *in vacuo* by 6 M HCl at 110°C for 24 h. Sulphur amino acids were determined after performic acid oxidation to methionine sulphone and cysteic acid.[25] Tryptophan was not determined.

3. Results

3.1. Kinetics of hydrolysis

The amount of free amino groups produced from gluten, glutenin and gliadin during peptic hydrolysis determined by reaction with ninhydrin are shown in Figure 2. Before hydrolysis gluten and glutenin fractions were only slightly soluble in hydrolysis buffer; a few seconds after addition of pepsin, they were readily solubilised indicating that peptic hydrolysis disrupted gluten and glutenin aggregates almost immediately generating molecules soluble at acid pH. More total amino groups were released by glutenin than by gliadin, but with all samples, the digestion reached half completion in about 40 min and appeared to be almost complete after 200 min. Increasing the pepsin to gluten ratio increased the total amount of free amino groups released (Figure 3).

SDS-PAGE patterns of purified gliadin peptic hydrolysates at various reaction times showed four major fractions A, B, C, D (Figure 4), the different composition of which can be seen from the

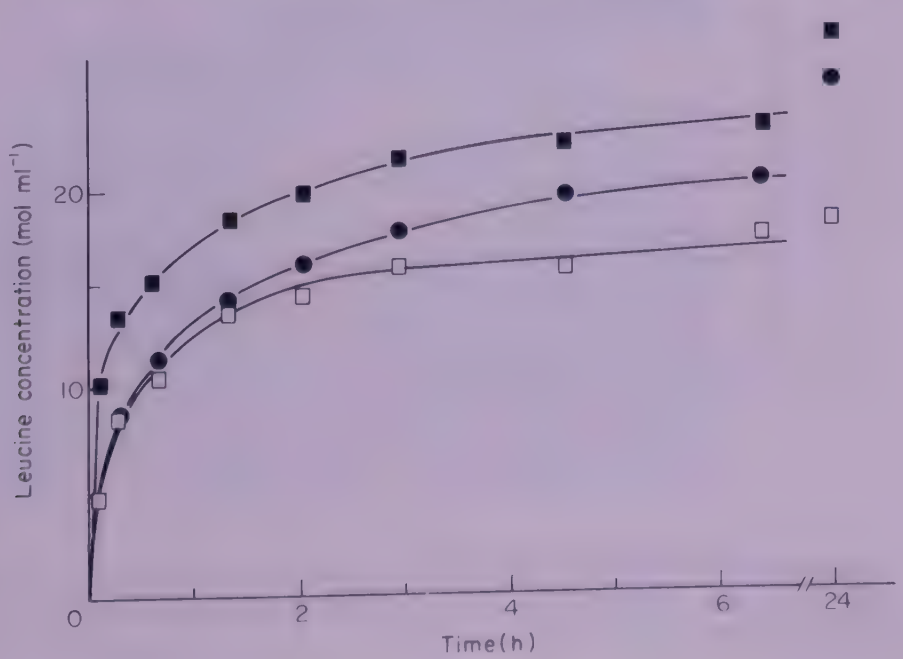

Figure 2. Production of terminal amino nitrogen during digestion of gluten, glutenin and gliadin fractions by pepsin (enzyme/substrate=1/33) at T=20°C and pH 2: (■), glutenin; (●), gluten; (□), gliadin.

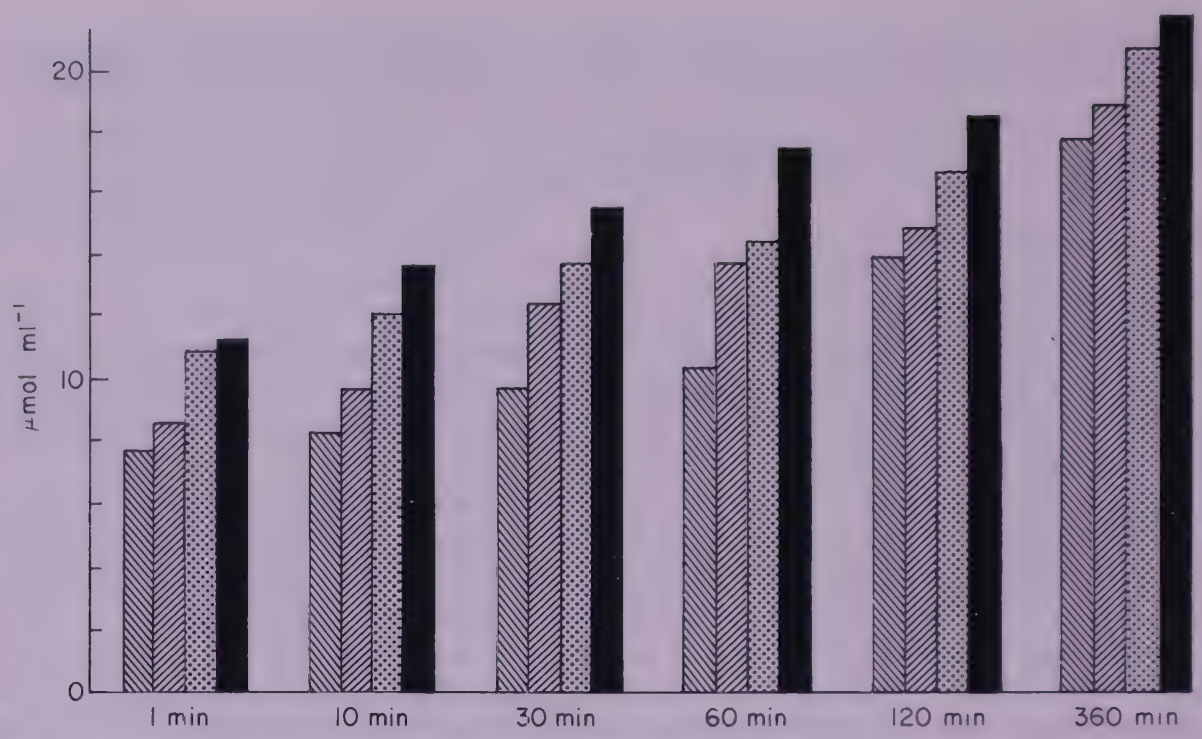

Figure 3. Influence of the concentration in pepsin [enzyme/substrate=(▨) 1/100, (▨) 1/50, (▨) 1/33 and (■) 1/20] on the amount of free amino groups produced from gluten. T=20°C, pH 2.

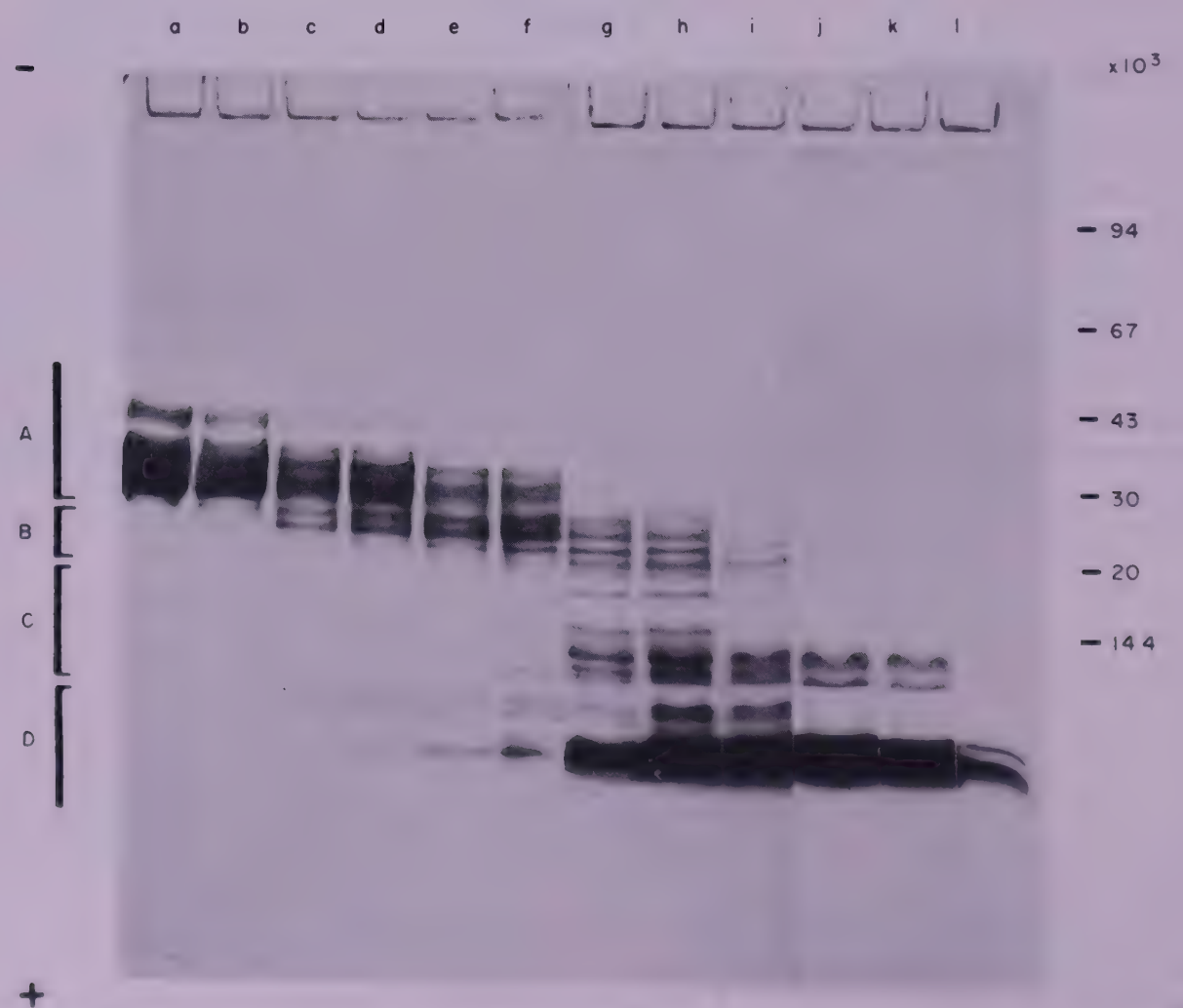

Figure 4. SDS-PAGE pattern of gliadin fraction for various time intervals. a, No enzyme control; b, 1 min; c, 3 min; d, 6 min; e, 10 min; f, 15 min; g, 40 min; h, 60 min; i, 120 min; j, 260 min; k, 380 min; l, 24 h. Hydrolysis was done at pH 2 and T = 20°C with an enzyme to substrate ratio of 1/33.

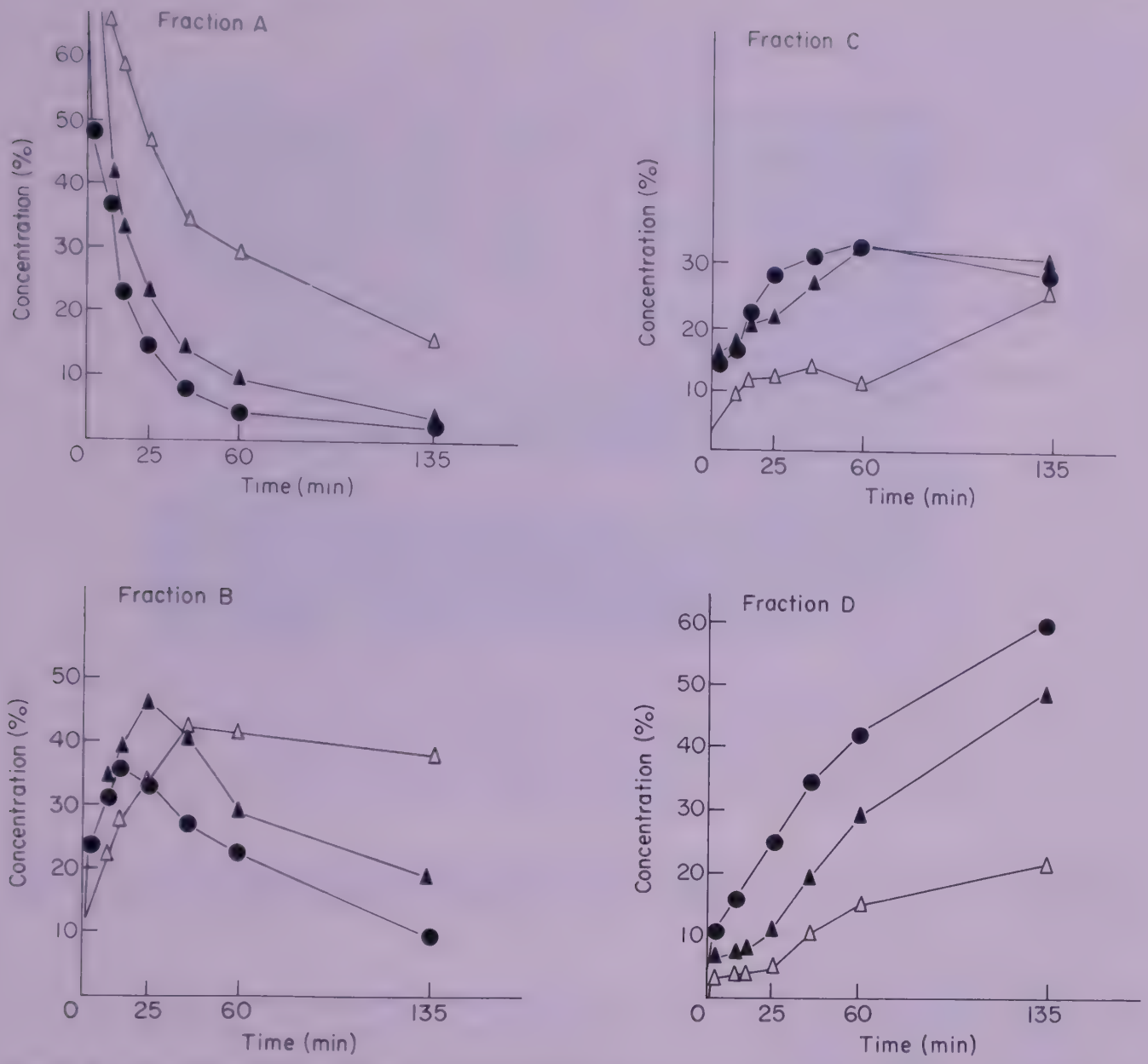

Figure 5. Variation with time of the different fractions resulting from an peptic hydrolysis of gliadin according to different enzyme to substrate ratios: (●), 1/33; (▲), 1/100 and (△), 1/1000. Fraction A, native gliadin; fraction B, polypeptides in the range 28 000–18 000; fraction C, polypeptides in the range 17 500–12000; fraction D, peptides <12 000. Hydrolysis was done at pH=2 and T=20°C.

densitometric patterns for various enzyme/substrate ratios (Figure 5). Increasing the enzyme to gliadin ratio increased the speed of the disappearance native gliadins (fraction A) but not the qualitative aspects of the electrophoretic pattern. Fraction B, which consisted mainly of peptides of MW in the range 28 000–20 000, reached a maximum between 15–45 min depending on the enzyme concentration. These components disappeared upon prolonged hydrolysates and resulted in peptides in the MW range 17 500–12 000 (fraction C) and lower than 9700 (fraction D).

SDS-PAGE patterns of glutenin peptic hydrolysates for various reaction times were more complex than those from gliadin (Figure 6), polypeptides appearing in the range 68000–65000, 34000–23 000 and 15000–9000. Further hydrolysis produced subunits of molecular weights near 24 000, and after 320 min components with MW>15 000 completely disappeared.

3.2. Fractionation of hydrolysates

Peptic hydrolysis of purified gliadin and crude glutenin was carried out for 2 h at 20°C with an enzyme/substrate ratio of 1/33, and the hydrolysates were chromatographed by gel filtration on Sephadex G50 column (Figure 7). Detection at 280 nm showed the presence of two major peaks for

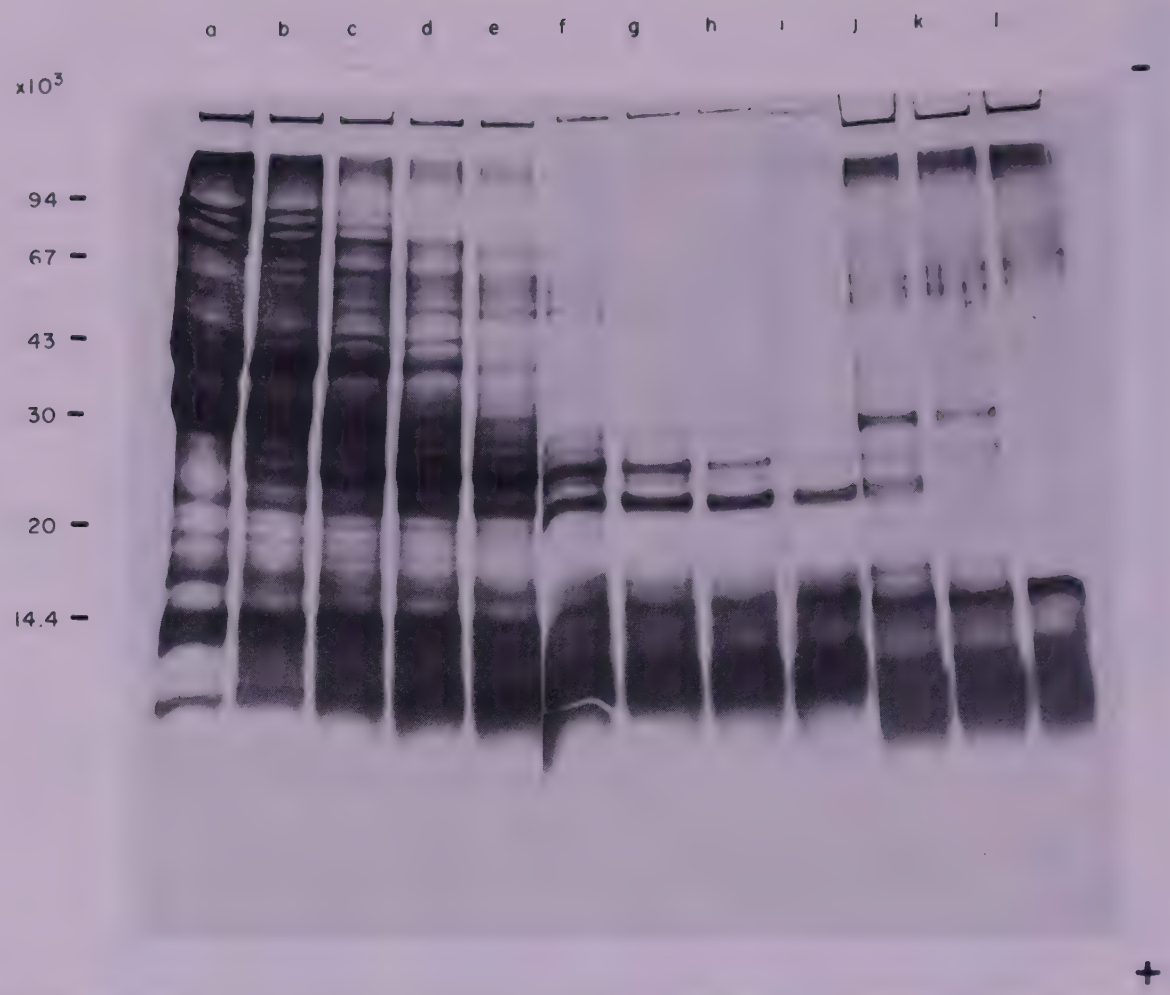

Figure 6. SDS-PAGE pattern of the glutenin fraction for various time intervals. a, non enzyme control; b, 1 min; c, 3 min; d, 6 min; e, 10 min; f, 25 min; g, 40 min; h, 60 min; i, 120 min; j, 260 min; k, 380 min; l, 24 h. Hydrolysis was done at pH 2 and $T=20°C$ with an enzyme to substrate ratio of 1/33.

gliadin hydrolysates (fraction 1 and 2) but only one for glutenin (fraction 1); however detection at 220 nm showed that both digests contained substances being continually eluted. These eluates were divided into six fractions which were then electrophoresed (SDS-PAGE) (Figures 8 and 9) and analysed for amino acid content (Table 1). The quantities of polypeptides recovered in each fraction, calculated from their amino acid contents, and their molecular weights obtained by gel filtration and SDS-PAGE are summarised in Table 2.

Fraction 1 of the gliadin hydrolysate comprised large polypeptides in the range 28 000–12 000 characterised by high contents of glutamate, proline and phenylalanine and smaller amounts of aspartate, sulphur containing amino acids, alanine, valine, isoleucine and leucine, compared to native gliadins. The relatively homogeneous fraction 2, which represented 41.5% of the total digest, consisted of components in the MW range 12 000–9000. Fractions 3 and 4 which presented apparent MW in the ranges 7500–2700 and 2700–1400 respectively on Sephadex G50, seemed similar to those in fraction 2 by SDS-PAGE but were weakly stained. Fraction 5 contained small peptides with MW under 1000 and fraction 6 (with an elution volume equal to the total volume of the Sephadex G50 column) is characteristic of components interacting with the gel. Fractions 5 and 6 did not react with the stain used after SDS-PAGE. From the amino acid analysis, fraction 3 was characterised by a high proline content; fractions 4 and 5 had compositions very different to that of gliadin.

With glutenin digests, the non-reduced fraction 1 showed large polypeptides with MW between 25 000 and 45 000 not found in native glutenin, moreover only one band had a MW<20 000. When the S–S bonds of fraction 1 were reduced many subunits <20 000 and some <14 000, were observed, only one band over 30 000 remaining. The amino acid composition of fraction 1 did not differ largely from the undigested protein but there were increased contents of glutamate and proline and a slight decrease in the amount of methionine and cysteine. The non-reduced fraction 2, characterised by the

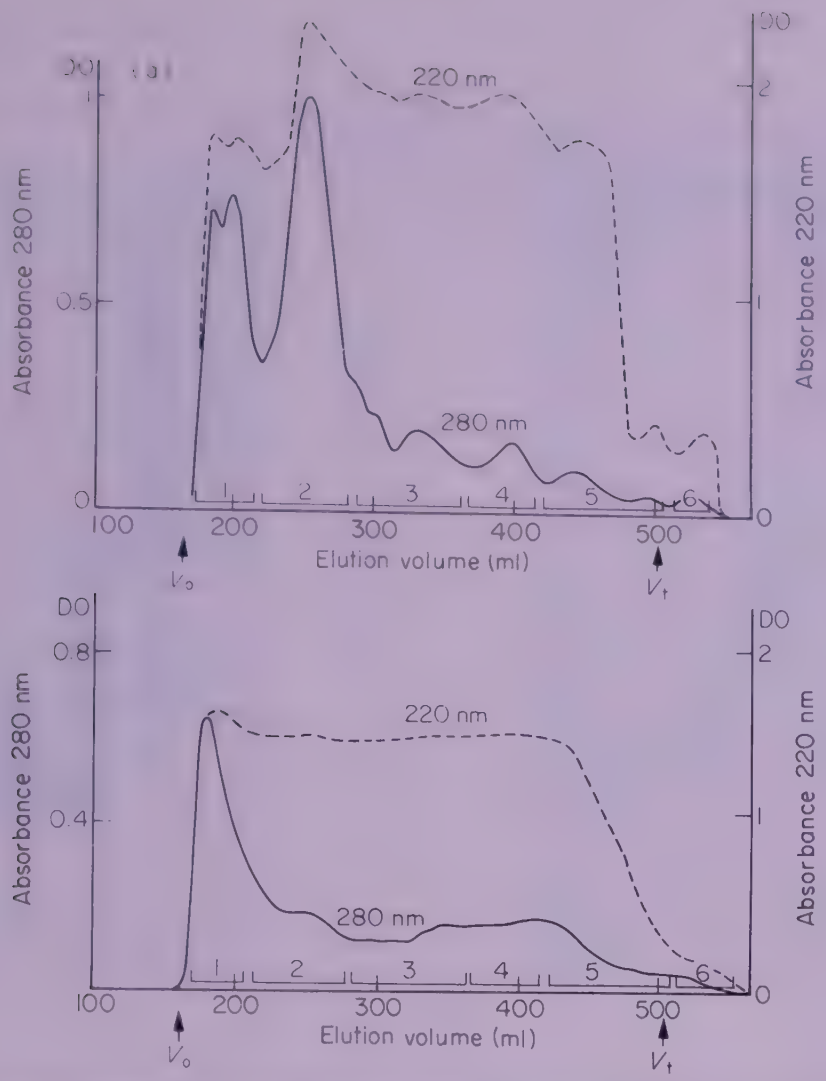

Figure 7. Chromatographic separations of peptic fragments from (a) gliadin and (b) glutenin fraction on a Sephadex G50 column. Conditions of digestion: 2 h at T=20°C in an enzyme to substrate ratio of 1/33.

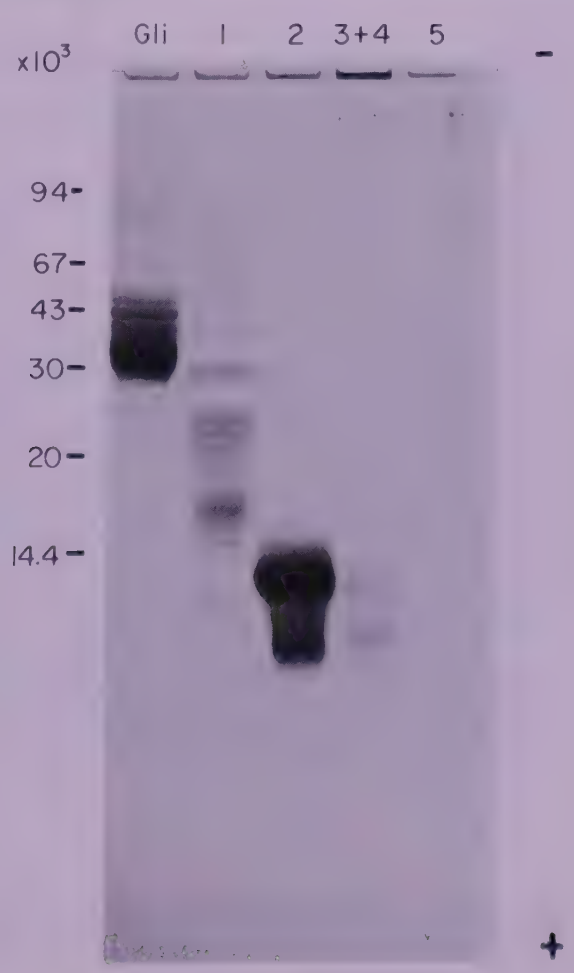

Figure 8. SDS-PAGE of the different fractions of gliadin obtained after gel filtration on Sephadex G50. Gli: native gliadin.

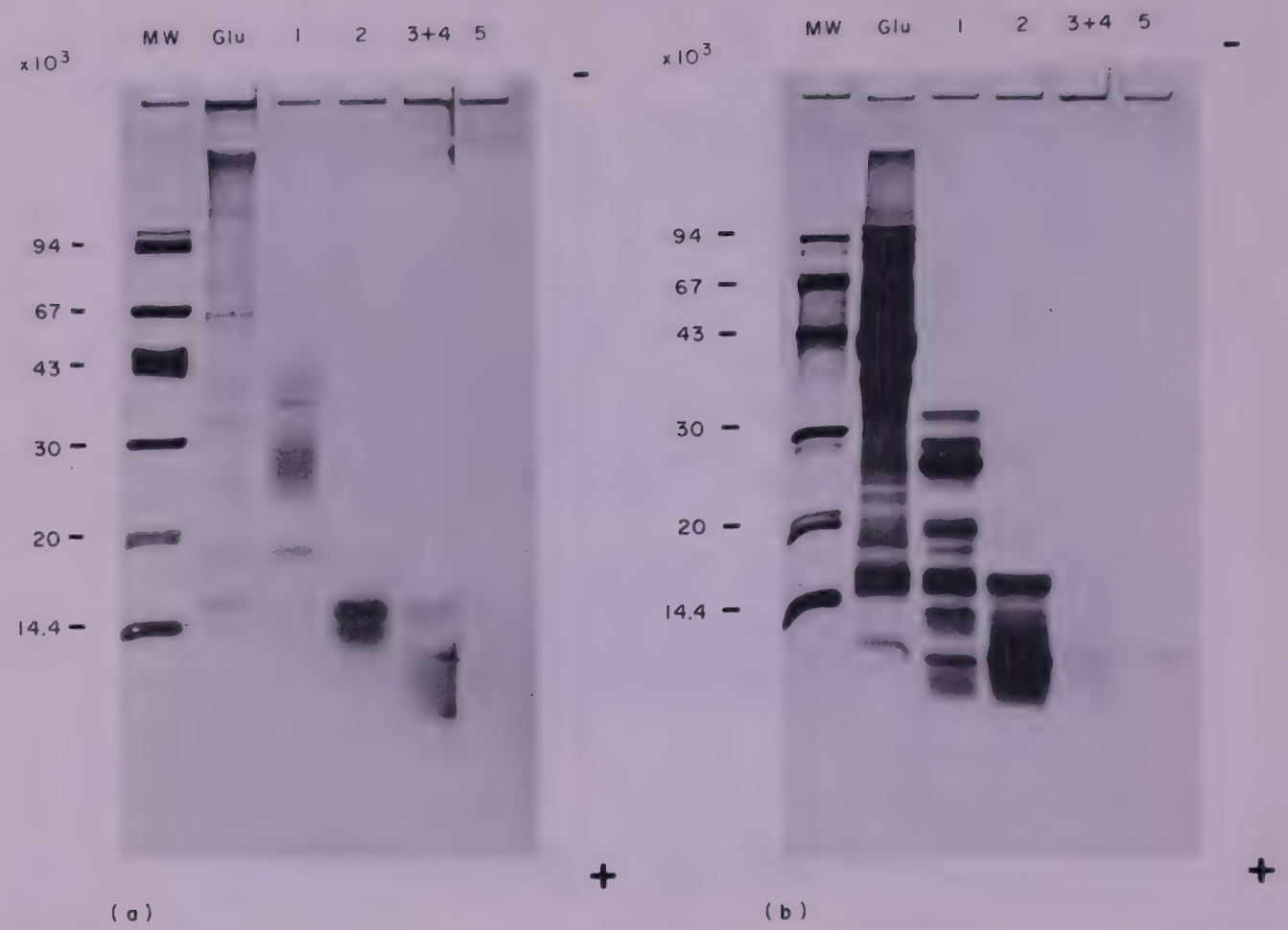

Figure 9. SDS-PAGE of (a) non reduced and (b) reduced fractions of glutenin obtained after gel filtration on Sephadex G50. Glu: native glutenin.

presence of two bands of approximately 15 000 produces many peptides of MW<15 000 (Figures 9a and 9b); after reduction only one band around 15 000 remained. The reduced and non-reduced fractions 3 and 4 possessed similar characteristics and fraction 5 stained only weakly.

Analysis by RP-h.p.l.c. of the six fractions from both the gliadin and glutenin digests separated by gel filtration on Sephadex G50 (Figure 10) showed the complexity of the fractions, even those which did not stain well. All fractions were soluble in TFA (0.05% (by vol) solution pH 2.5) except the native glutenin which was reduced before injection. According to their retention time on the C18 column no gliadin peptide displayed a surface hydrophobicity higher than that of native gliadin and as the MW of the peptides decreased, retention time also decreased. Furthermore, the peptide patterns appeared more complex, covering a larger range of hydrophobicities. The same pattern was observed with glutenin digest fractions. Fractions of a given molecular weight displayed the same range of hydrophobicity as the corresponding gliadin digest fraction, except for the first one eluted from Sephadex G50. This fraction, comprising the largest peptides was more heterogeneous in glutenin digests but less so in gliadin digests. Glutenin digest fraction 1 was composed of two groups of peptides, the first was eluted between 26.5 and 38.5% acetonitrile, and corresponded to the range of surface hydrophobicity of peptides contained in gliadin digest fraction 1. The second group was eluted between 38.5% and 54% acetonitrile, and corresponded, as far as surface hydrophobicity was concerned, to the less hydrophobic glutenin subunits or native gliadin. But in contrast to native glutenin these peptides were readily soluble in 0.05% (by vol) TFA.

4. Discussion

The amount of free amino groups produced from gluten and its components, glutenin and gliadin, during peptic hydrolysis confirmed previous results that wheat proteins possessed peptic sensitive

Table 1. Amino acid composition (as residues ‰) of the different fractions after a gel filtration on Sephadex G50 of an peptic digest (2 h, E/S=1/33, t=20°C) of glutenin and gliadin.

		Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
Gliadin						
Asp	27	17	25	21	39	99
Thr	16	27	15	9	12	27
Ser	42	33	37	50	59	60
Glu	427	455	453	462	408	273
Pro	149	230	144	170	76	80
Gly	16	17	11	25	41	64
Ala	20	12	13	15	27	38
½Cys	28	12	28	10	31	29
Val	37	16	33	36	42	33
Met	17	4	14	5	30	7
Ile	36	16	34	22	57	56
Leu	70	39	63	61	100	85
Tyr	20		28	23	9	33
Phe	46	84	54	55	14	26
Lys	5	7	7	5	14	30
His	21	15	19	15	15	
Arg	23	16	22	16	28	60
Glutenin						
Asp	48	16	41	54	77	87
Thr	31	23	23	25	37	31
Ser	52	54	49	52	58	47
Glu	328	419	346	302	227	139
Pro	112	133	123	105	60	6
Gly	40	50	33	47	52	55
Ala	31	17	26	42	56	48
½Cys	27	23	25	11	3	9
Val	41	30	50	53	62	51
Met	22	16	14	13	9	18
Ile	34	25	39	38	56	60
Leu	69	56	78	78	95	62
Tyr	31	34	26	30	28	132
Phe	45	43	38	52	52	118
Lys	25	10	16	30	38	33
His	22	22	25	22	26	33
Arg	42	29	48	46	64	71

[a] Fraction 6 (representating 0.1% of the total digest) was not analysed.

bonds.[4, 6] Digestion was rapid with a five-fold increase of free amino groups in 2 h with gluten. When the enzyme to substrate ratio was increased free amino groups increased but in lower proportions.

Variation of the pepsin/gliadin ratio did not modify the nature of the peptides produced but altered the kinetics of the digestion process that took place sequentially. When the enzyme concentration was lowered to 1/1000 or 1/100 the appearance of fraction D was considerably retarded although fraction A was extensively degraded and fractions B and C had increased. Our interpreta-

Table 2. Weight distribution of polypeptides and peptides separated by gel filtration on Sephadex G50 of peptic digests of glutenin and gliadin (E/S=1/33, 2 h at t=20°C) according to yield calculated from amino acid analysis

	Fraction					
	1	2	3	4	5	6
Apparent range of MW (daltons)	18000	18000 — 7500	7500 — 2700	2700 — 1400	<1400	Interaction with gel
Gliadin yield (%)	20.8	41.5	31.0	4.4	22	0.1
Glutenin yield (%)	25.7	21.2	29.6	22.2	1.2	0.1

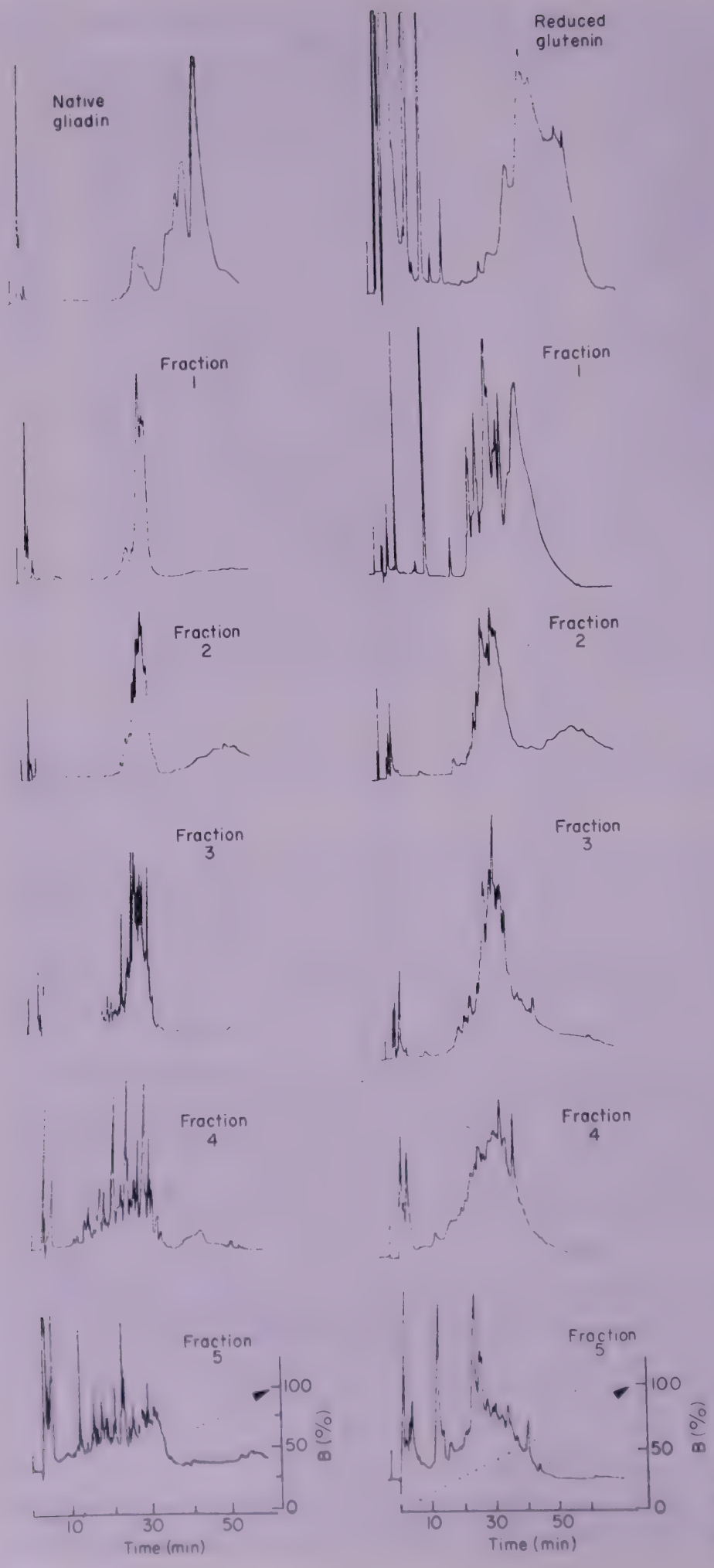

Figure 10. H.p.l.c. separations of peptic fractions from gliadin and glutenin fractions on an ODS reversed phase C18 column, after gel filtration on Sephadex G50 column.

tion is that, during the first stage of hydrolysis gliadin proteins were cleaved essentially in two families of fragments: one of about 25 000 (fraction B) and the other of approximatively 15 000 (fraction C). However the largest increase of fraction C corresponded to a decrease of fraction B, suggesting that fraction C also contained some degradation products of fraction B. Lastly, fraction D corresponded to the small peptides coming from various hydrolysis steps of the other fractions.

Large polypeptides with MW in the range 28 000–20 000 produced in the first stage can constitute up to 45% of gliadin digest after 45 min. They were characterised by high contents of glutamate, proline and phenylalanine. Although this fraction was composed of peptides coming from several gliadin components, its composition was related to the amino acid sequence of the α/β type gliadins taken as reference proteins.[26] Their sequence can be divided in domains, one of them (domain II) being characterised by a sequence repetition with high proline and glutamate contents whereas other domains contained lower amounts of proline.[26] So it is probable that large peptides are derived from native gliadins by cleavage of the polypeptides chain in its —COOH terminal region because of proline enrichment and decreased cysteine content. Moreover a lower cysteine content suggests that cleavage occurred near the cys–cys doublet (156–157). Cleavage at this site would generate a large peptide of MW 20 000 and a smaller one around 13 000.

The results here show that peptic hydrolysis of glutenin produced firstly large polypeptides in the range 68 000–65 000 and 34 000–23 000, which were rich in glutamate and proline, and were soluble in the different non-denaturing solvents used. Their formation was accompanied by a rapid solubilisation of samples and a decrease of the viscosity probably due to cleavage in interchain disulphide bond regions.[6, 10] This hypothesis, which disagrees with other published results,[12] was strengthened in the present study by the observed presence of peptides containing interchain disulphide bonds.

The second stage of hydrolysis was characterised by the appearance of components of MW around 15 000 for gliadin and 24 000 for glutenin and having amino acid compositions similar to those of the native proteins. Some of the subunits derived from glutenin were also S–S branched peptides.

The final stage of hydrolysis produced peptides of MW<10 000. Degradation of these small peptides cannot be described with precision because of the complexity of the mixture. They could represent an important part, nearly 50%, of the digest in the case of prolonged hydrolysis. The free amino acids were present in low concentration and accounted for less than 1.2% and 3.2% for gliadin and glutenin digests respectively. Average molecular weights of 1150 daltons for glutenin peptides and 1990 for gliadin peptides were calculated after complete hydrolysis of the sample.[10]

Glutenin and gliadin digests fractions were composed of polypeptides differing in molecular weight, amino acid composition, surface hydrophobicity and structure. These results confirmed previous observations of differences in polypeptide size.[10] This might be due either to differences in amino sequences of gliadin and glutenin, or in protein conformation. These results showed that differences of site location, structure and conformation could explain differences in hydrolysis and of peptide characteristics previously described.[10–12] Production of large peptides in the early stage of gliadin digestion suggests that most of peptic cleavage sites were not accessible in the native molecule. Conversely the initial digestion of glutenin leads to a rapid decrease of the molecular weight of components. Moreover, according to the kinetics data, glutenin was hydrolysed more rapidly than gliadin. After 10 min (enzyme/substrate=1/33) intact glutenin subunits had totally disappeared, whereas up to 20% of native gliadins were still present. Thus hydrolysis sites were probably more accessible to enzyme attack in glutenin than in native gliadin. These results can be related to viscosimetric studies that showed that at pH 2.9–9.0 in 0.1 M acetic acid a gliadin molecule retained a certain degree of compactness although a part of the molecule was probably expanded. Conversely if the structure of glutenin in those conditions is of that proposed by Tatham *et al.*[27] these macromolecules could be more accessible to peptic digestion.

It has been previously reported[12, 22] that pepsin-sensitive bonds are principally located in terminal regions of polypeptide chains. Those findings were confirmed here with gliadins at the first stage of digestion where results indicated that only a part of —COOH terminal region was cleaved. Modification of SDS-PAGE pattern of glutenins after reduction (Figure 9) indicated the presence of the disulphide branched polypeptides in the hydrolysate. As it has been shown that most cysteine

residues are located very near the —COOH and —NH2 terminal ends of chains,[28–32] peptic cleavage might not occur in the very terminal region of subunits because S–S reduction of branched peptides leads to a decrease of their MW from 40 000 to 20 000.

As the complexity of native protein samples and of peptidic patterns hindered definite conclusions about the sequential appearance of peptides and location of hydrolysis sites, and also renders peptide purification difficult for biochemical and functional characterisation, studies are in progress on highly purified gliadin components.

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The Cytochrome c Content of Various Poultry Meats

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The cytochrome c content and total haem pigments in the breast and thigh meat of broiler chickens, hens out of lay, turkeys, geese, duckling (Peking), and muscovy ducks were determined. The levels of cytochrome c in the breast and thigh meat were: for chicken 11.4 and 35.5; for hens 14.0 and 84.2; for turkeys 13.1 and 47.4; for geese 141.0 and 107.8; for duckling 111.2 and 149.9 and for muscovy ducks 81.5 and 97.3 μg per g of tissue, respectively. The correlation coefficient between cytochrome c and total haem pigments concentration was $r=0.98$ for breast, and $r=0.85$ for thigh meat. The authors postulate that the cytochrome c may play a noticeable role in the colour of fresh and processed poultry meat, but further investigations are needed to confirm this.

Keywords: cytochrome c; total haem pigment; poultry meat.

1. Introduction

Cornish and Froning[1] considered that cytochrome c played some role in the colour stability of turkey meat. The autoxidation rate of turkey myoglobin exhibited a six-fold slower rate in the presence of cytochrome c. The authors found that cytochrome c resists heat denaturation at 105°C. Ngoka and Froning[2] reported that the darker colour in breast muscle from turkey exposed to excitement before slaughter and allowed to struggle freely during slaughter may be due to a higher concentration of cytochrome c. The authors suggested that cytochrome c may contribute to the pink colour problem in processed turkey products. Cheah[3] found that the initial level and decrease in the cytochrome c content of the mitochondria isolated from the *longissimus dorsi* muscle of pigs could be attributed mainly to differences in initial pH values and in the subsequent postmortem rates of pH decline. Cheah and Cheah[4] also observed a decrease in cytochrome c content of mitochondria isolated from ox neck muscle during postmortem storage of muscle, in spite of its high initial and ultimate pH values. Drabkin[5] noticed that cytochrome c is present at a concentration of approximately 10 to 50×10^{-3} μmol g^{-1} of skeletal muscle, while the myoglobin content is 50 to 440×10^{-3} μmol g^{-1} of skeletal muscle. Unfortunately these data refer to such species as rats, horses and dogs.

In the available literature no information was found on the cytochrome c concentration of poultry meat. It was necessary therefore, to study the content of cytochrome c in various poultry meats and its relation to total haem pigments.

2. Materials and methods

2.1. Experimental materials

The birds were slaughtered in a commercial processing plant by standard procedures for different types of poultry. After evisceration and chilling in ice water the carcasses were immediately taken to the laboratory. The breast and thigh muscles were separately excised, comminuted, minced, wrapped in aluminium foil, frozen and then stored at −18°C until used for analysis. The samples for analysis were cut into small flakes directly from the frozen meat. The weight of these samples varied

from 20 g for breast and thigh meat of waterfowl and thigh meat of hens; 30 g for thigh meat of broiler chicken and turkey, 60 g for breast meat of landfowl.

2.2. Determination of haem pigments

2.2.1. Total haem pigments

Total haem pigments, including the cytochrome c, were extracted from meat twice with a 0.04 M phosphate buffer (pH 6.8) using the method of Warriss.[6] They were then homogenised for 60 s, centrifuged for 10 min at $4000\times g$, and supernatants were clarified by additional centrifuging for 1 h at $30\,000\times g$. Absorbance was measured at 525 nm without the conversion of pigments to their cyanmet forms.[7, 8]

2.2.2. Cytochrome c concentration

The cytochrome c determination was performed according to the procedure of Izumi *et al.*[9] 90% of the extract volume for total haem pigments analysis was adjusted to pH 3.0 with 4 M HCl, held for 1 h, and the pH was then adjusted to 7.0 with 4 M NaOH. In these conditions the myo-, and haemoglobin were precipitated. The precipitated proteins were removed by centrifuging for 5 min at $4000\times g$, and the supernatant was filtered through Whatman No. 1 paper. The clarified filtrate was concentrated about 10-fold under reduced pressure at 40–50°C. The solution was again centrifuged for 10 min at $12\,000\times g$, filtered through Whatman No. 1 paper and the volume of the condensed extract was measured. For reduction, to one part of extract solid sodium dithionite, and for oxidation solid potassium ferricyanide were added. The absorbance at 550 nm of oxidised and reduced forms of cytochrome c was measured.

The concentration of cytochrome c was calculated from the difference of absorbance between the reduced, pink coloured, and oxidised yellow coloured forms:

$$\text{Cytochrome c content} = \frac{(A_{red} - A_{oxid}) \cdot 1284 \cdot V}{19\,100 \cdot C} \text{ mg g}^{-1}$$

A_{red} is the absorption of reduced cytochrome c, A_{oxid} is the absorption of oxidised cytochrome c, 1284 is the molecular weight of cytochrome c (type VI, from horse heart), V is the coefficient of dilution (cm^3), 19 100 is the molar extinction coefficient for reduced–oxidised forms of cytochrome c (Potter and DuBois)[10] and C is the weight of meat sample (g).

To the thigh meat of chicken, containing 35.5 ± 1.12 μg cytochrome c g^{-1} of tissue, was added 30.9 μgof oxidised cytochrome c reagent g^{-1} of meat sample purchased from Sigma Chemical Co. After analysis the recovery of cytochrome c was found to be 95.8 ± 0.48%. Statistical analysis of total haem pigments and cytochrome c contents were performed by the two-tailed Student's *t*-Test.[11]

3. Results and discussion

3.1. Total haem pigments in various poultry meats

The data on haem pigment content should be attributed to definite types or groups of birds. The relevant characteristics of birds and carcasses used in this experiment is given in Table 1. The total haem pigment content found in poultry meat is presented in Table 2. The lowest concentration was found in broiler chicken muscles, 0.49 and 1.66 mg g^{-1} of tissue and the highest in goose muscles, 6.53 and 3.90 mg g^{-1} in the breast and thigh meat, respectively. However, the slightly higher level of pigment found in the breast meat of hens and turkeys rather than in chickens was not significant.

The landfowl showed less pigment than the waterfowl, especially in the breast muscle. The thigh muscles of landfowl contained much more haem pigment than their breast muscles, whereas the waterfowl showed significantly more pigment in breast than in thigh meat. Generally these results are very close to previous investigations on the haem pigment content in poultry meat[8] but additionally, they show the total haem pigment in muscovy ducks. Some differences in thigh meat of hens and turkeys may be attributed to the age of the birds. The content of haem pigments in the same muscles of the same type of bird vary depending on the age, sex and even strain as well as analytical

Table 1. Characteristics of the material used in experiment

Type of poultry	Weight of carcasses (g)	Age of birds (weeks)	Sex	pH values[a]	
				Breast meat	Thigh meat
Broiler chicken	1186±24.4	8	Male and female	6.0±0.03	6.6±0.04
Hens out of lay	2404±45.4	62	Female	6.0±0.04	6.6±0.05
Turkeys	5114±108.3	17	Female	6.0±0.06	6.6±0.06
Geese	2972±67.9	16	Male and female	6.1±0.05	6.7±0.04
Duckling (Peking)	1505±39.6	8	Male and female	6.0±0.03	6.7±0.05
Muscovy ducks	1488±52.1	10	Female	6.1±0.04	6.7±0.04

Data are presented as means±the standard deviation from six carcasses.

[a]The pH value was measured at the moment when samples of meat were taken for haem pigment analysis, average 24 h after slaughter.

Table 2. Content of total haem pigments in breast and thigh meats in various types of poultry

Type of poultry	Haem pigments, mg g^{-1} of tissue	
	Breast meat	Thigh meat
Broiler chicken	0.49 ± 0.058^{aA}	1.66 ± 0.97^{aB}
Hens out of lay	0.64 ± 0.046^{aA}	3.06 ± 0.125^{cdB}
Turkeys	0.58 ± 0.052^{aA}	2.15 ± 0.112^{bB}
Geese	6.53 ± 0.235^{dB}	3.90 ± 0.168^{cA}
Duckling	4.31 ± 0.165^{cB}	3.54 ± 0.105^{deA}
Muscovy ducks	3.57 ± 0.163^{bB}	2.54 ± 0.124^{bcA}

Data are presented as means±the standard deviation from six carcasses and three parallel determinations.

Figures in the columns (a–e) and in the rows (A–B) marked with different letters are significantly different at $P=0.05$.

method.[8,12–14] The total haem pigments content presented in this paper appear a little higher than values given by other workers, mainly due to the different analytical procedure. In our procedure meat samples were frozen before comminuting and phosphate buffer was used instead of water for extraction. These conditions significantly improved the extractability of the total haem pigments.[8]

3.2. Content of cytochrome c in various poultry meats

The content of cytochrome c in the breast and thigh meat of various types of poultry are summarised in Table 3. The cytochrome c concentration in poultry meat showed a similar pattern to the total

Table 3. Content of cytochrome c in breast and thigh meats in various types of poultry

Type of poultry	Cytochrome c, $\mu g\ g^{-1}$ of tissue	
	Breast meat	Thigh meat
Broiler chicken	11.4 ± 0.55^{aA}	35.5 ± 1.12^{aB}
Hens out of lay	14.0 ± 0.69^{aA}	84.2 ± 1.98^{cB}
Turkeys	13.1 ± 0.49^{aA}	47.4 ± 1.47^{bB}
Geese	141.0 ± 3.37^{dB}	107.8 ± 3.21^{deA}
Duckling	111.2 ± 2.87^{cA}	149.9 ± 4.33^{fB}
Muscovy ducks	81.5 ± 2.31^{bA}	97.3 ± 2.48^{dB}

Data are presented as means±the standard deviation from six carcasses and three parallel determinations.

Figures in the columns (a–f) and in the rows (A–B) marked with different letters are significantly different at $P=0.05$.

haem pigment content, although the absolute values are very much lower. In geese, however, the breast meat contained significantly less cytochrome c than the thigh muscles, and landfowl in turn contained less than waterfowl. The highest content of cytochrome c was found in the breast meat of geese (141.0 $\mu g\ g^{-1}$), and the lowest in the breast of chicken (11.4 $\mu g\ g^{-1}$). Referring to the paper of Cheah,[5] it was found that, except for geese, breast muscles with lower pH values (6.0–6.1) at the moment of sampling for haem pigment analysis, contained less cytochrome c than thigh meat with higher pH values (6.6–6.7).

3.3. The percentage of cytochrome c in total haem pigments

The detailed data on the percentage of cytochrome c in the total haem pigment content of poultry meat are presented in Table 4. It is interesting to observe that, in spite of differing contents of total haem pigments in the breast muscle, the percentage of cytochrome c to haem pigments remained almost constant, at a mean level of 2.35%, whereas in the thigh muscles some significant differences were found. This was especially the case with the thigh meat of duck, where cytochrome c constituted about 4% of total haem pigments. In the breast meat of all poultry and in the thigh meat of chickens, hens, turkeys and geese the level of cytochrome c is about 40 times less than total haem pigments, while in the thigh meat of duckling and muscovy ducks it is about 1 to 25. The correlation coefficient between the cytochrome c and total haem pigment in breast meat of all investigated poultry is as high as $r=0.98$. The same high correlation also exists when taking into account the thigh meat of landfowl and geese. In the case of all thigh meat the correlation coefficient between cytochrome c and total haem pigments was $r=0.85$. This lower correlation was due to the higher content of cytochrome c in duck thigh muscles.

Table 4. The percentage of cytochrome c to total haem pigments content in breast and thigh meats of various types of poultry

Type of poultry	Breast meat	Thigh meat
Broiler chicken	2.33 ± 0.055^{aA}	2.14 ± 0.086^{aA}
Hens out of lay	2.22 ± 0.071^{aA}	2.75 ± 0.098^{aA}
Turkeys	2.36 ± 0.067^{aA}	2.20 ± 0.081^{abA}
Geese	2.16 ± 0.101^{aA}	2.76 ± 0.103^{bB}
Duckling	2.58 ± 0.098^{aA}	4.23 ± 0.121^{cB}
Muscovy ducks	2.28 ± 0.081^{aA}	3.83 ± 0.110^{cB}

a–c, and A–B, see Table 2.

3.4. The supposed role of cytochrome c in the colour of poultry meat

As was suggested by authors cited above[1,2] cytochrome c may play a role in the colour of fresh and processed poultry meat. The significant differences in cytochrome c content between the types of poultry meat observed in these investigations support these suggestions. The reduced form of cytochrome c which is pink coloured is most desirable for meat while the oxidised form is yellow. Izumi *et al.*[9] suppose that cytochrome c may react with nitrite in cured meat if ascorbate or erythorbate is used. Heat resistance of cytochrome c to coagulation should also be taken into consideration, because, raw meat loses its red colour during cooking due to the decomposition of myoglobin and haemoglobin, while native and stable cytochrome c may retain its natural colour during heating of meat up to 105°C.[1]

Because of a poor knowledge of the properties of cytochrome c in meat and of its reaction during processing with the components of meat and meat additives, it is impossible to monitor the colour of meat on the basis of cytochrome c content but the problem seems to merit further research.

4. Conclusion

The content of cytochrome c in landfowl varied from 11.4 to 14.0 in the breast and from 35.5 to 84.2 $\mu g\ g^{-1}$ of tissue in the thigh meat, respectively. However in the waterfowl the respective values were;

81.5 to 141.0, and 97.3 to 149.9. The cytochrome c constituted from 2.16 to 4.23% of total haem pigments in various poultry meats. The higher values were in the thigh muscles of ducks. Cytochrome c may have a role to play in the colour of poultry meats; its importance needs to be assessed.

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